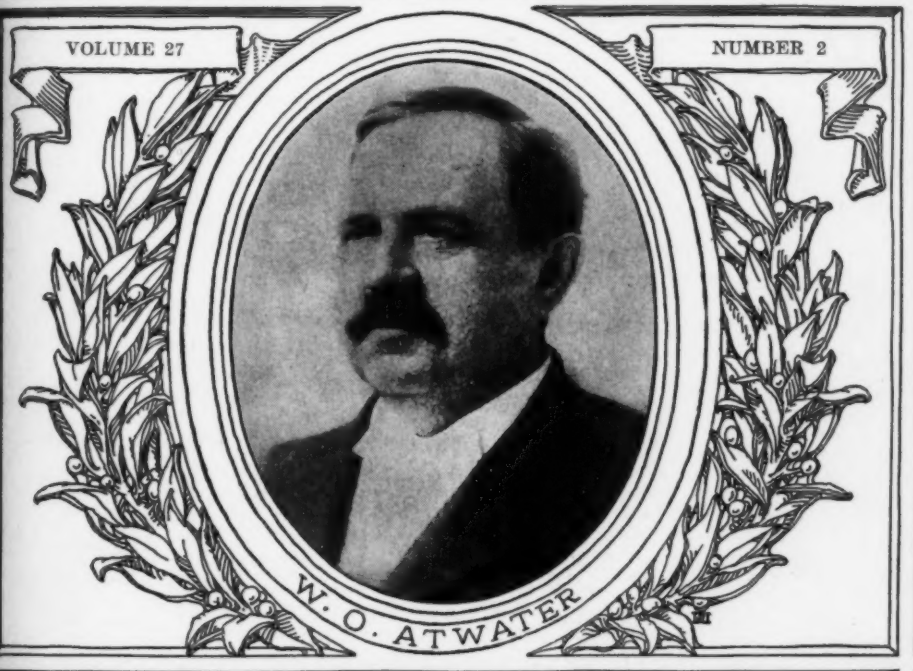
FEBRUARY 10, 1944

THE JOURNAL OF NUTRITION

VOLUME 27 NUMBER 2



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Official Organ of the American Institute of Nutrition
PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA 4, PA.
Price, $5.00 per volume, Domestic; $5.50 per volume, Foreign

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NUTRITIONAL DERMATOSES IN THE RAT

IX. EVALUATION OF THE INTERRELATIONSHIP OF MAGNESIUM DEFICIENCY AND DEFICIENCIES OF THE VITAMIN B COMPLEX [1]

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ONE PLATE (ELEVEN FIGURES)

(Received for publication August 20, 1943)

Deficiency of the vitamin B complex has been considered a factor which influences the signs of magnesium deficiency (Watchorn and McCance, '37; Greenberg and Tufts, '38). In this communication the signs of "uncomplicated" magnesium deficiency are described and the question of the interrelationship of the deficiencies of the vitamin B complex and magnesium deficiency is evaluated.

EXPERIMENT I

"UNCOMPLICATED" MAGNESIUM DEFICIENCY

For the production of "uncomplicated" magnesium deficiency the following diet was employed: vitamin-free [2] casein, 200 gm.; dextrin, 541 gm.; butter fat, 100 gm.; McCollum salts no. 62 (Kruse, Orent and McCollum, '32) 59 gm. Crystalline supplements of 20 mg. of thiamine hydrochloride, 10 mg. of riboflavin, 10 mg. of pyridoxine, 30 mg. of nicotinic acid, 15 mg. of calcium pantothenate, 1 gm. of choline hydrochloride, 375 mg. of inositol, and 375 mg. of para-amino-benzoic acid were incorporated into the diet. Thiamine hydrochloride, riboflavin, calcium pantothenate, pyridoxine, and nicotinic acid were calculated on the basis of the amounts of these vitamin B complex factors which are contained in 100 gm. of yeast [3] of known assay (Sullivan and Evans, '43). Five hundred milligrams of cystine and 18,200 I. U. of vitamin A, and 5,460 I. U. of vitamin D [4] were included in the ration.

[1] Aided by a grant from the Rockefeller Foundation Fluid Research Fund.

[2] S. M. A. Corporation.

[3] Anheuser-Busch.

[4] Mead Johnson's Oleum Percomorphum.

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Each animal received supplements of 10 mg. of alpha-tocopherol per week. The diet was planned to include all of the known essential aliments and was considered to be otherwise adequate except for magnesium, of which it was calculated to contain less than 1.8 mg. in each kilogram. The control diet differed from the deficient diet in that 1 gm. of $MgSO_4.7H_2O$ was triturated into the salt mixture and was added at the expense of 1 gm. of dextrin to each kilogram of the diet.

Two or 3 days after the experimental ration was fed to recently-weaned rats the animals were nervous and hyperirritable. Convulsive seizures occurred spontaneously and were precipitated by noise or handling any time after the first week. The average duration of the convulsive seizures was 2 minutes; it varied from 1 to 14 minutes. Some of the animals succumbed immediately after the convulsions; others survived to experience several subsequent seizures. In twenty-nine litters consisting of 200 animals, eleven were observed at various times to have experienced at least three convulsive seizures and it is likely that at unobserved times, especially at night, additional seizures occurred. The tonic-clonic convulsive seizure has been described by several investigators since it was first reported by McCollum and Orent ('31). The animal races wildly around the cage in great confusion. It falls on its side and is extremely spastic in the first stage of the seizure; the eyes are closed tightly; the extremities are hyper-extended stiffly for several seconds, after which the eyelids gradually open, the animal relaxes, and the extremities are flexed rapidly. This stage is usually followed by a stage in which the animal is again rigid and opisthotonic; the animal then lies in a semi-somnolent state; it is listless, flaccid, and clumsy for a few minutes before it recovers or dies.

The first cutaneous sign was erythema; it occurred on the fourth to the eighth day, depending upon the litter. As a rule, the first sites involved by erythema were the trunk and/or the conchae of the ears. Again this depended upon the litter as the implicated sites were uniform for each group of animals. Erythema spread rapidly and on the trunk it was generalized in some animals and/or distributed in numerous non-grouped multiform plaques in others. From the conchae the erythema spread peripherally to the tips of the ears and it was usually of an intensity that suggested hemorrhage (fig. B). Shortly afterwards the areas involved by the erythematous plaques and the diffuse erythema were edematous. In the case of the ears this was striking as they usually were swollen to approximately twice their normal thickness (fig. B). Coincident with or soon after the initial cutaneous signs the paws were swollen and red. The edema and erythema

of the paws were not preceded or accompanied or followed by scaling. During the erythematous stage the animals continued to manifest signs of irritability. Convulsive seizures occurred frequently but not necessarily in the erythematous stage. The initial stage of erythema lasted as a rule from 4 to 7 days, but in some litters the stage was shorter; it was followed by a period during which there was pallor. Hyperirritability continued for some time after the erythema subsided. There was no change in the lustre of the fur and alopecia was not observed in the early stage.

Growth during the first 2 or 3 weeks approximated that of rats in the control groups. Thereafter there was less rapid rate of growth. In animals that survived for 3 months the increment of weight was 100% and the peak of the weight curve was reached between the eighth and the tenth week. Thereafter until death there was a slight decrease in weight.

As the disease progressed the animals were weak and less irritable. In the late stage the animals were listless and reluctant to be handled; their behavior was of a sullen type. Priapism occurred in the majority of male animals. Although spontaneous convulsions were infrequent in the late stage, they occasionally occurred and were induced a few times. In three litters there was widespread, diffuse alopecia of the venter. Kruse, Orent and McCollum ('32) observed alopecia in the late stage of the deficiency. It has been emphasized before that the signs and symptoms in a late stage of a deficiency are unimportant insofar as they aid in evaluating specific effects of a deficiency. This is especially true in the magnesium-deficient rat because after the sixth to the eighth week there is nutritive failure and there are extensive internal changes (nephritis, hepatitis) which vary in different rats and which predispose to superimposed illness, as well as contribute to the general state and symptoms of the sick animals. Occasionally there was hair loss which was confined to individual plaques of eczematous dermatitis that occurred at the sites previously involved in the early stage by the erythematous, edematous plaques. In some cases there was a crusting and scaling of the margins of the ears (fig. C) and in many animals the ears remained thickened until death. In no instance was there distortion of the contour of the ears. Scattered plaques of dermatitis involved every portion of the skin of some animals. In the tails of a few there were extensive changes which included dermatitis, induration, ulceration and gangrene. Frequently after 8 weeks there were ulcers on the plantae. A characteristic sign of the deficiency was the manner in which the skin of the tail was detached

from the fibrous tissue immediately surrounding the bone of the tail. When an animal was suspended by the tail, the skin slipped from its attachment to the deeper structure. By gently rotating the tail between the thumb and forefinger, this phenomenon of slippage was readily demonstrated. During the late stage the skin was thickened and tougher; when it was cut with a knife it offered more resistance than the skin of control animals. The skin lost its resiliency. If the skin of the back was grasped between the thumb and the forefinger and lifted into folds it failed to "snap back" rapidly into place after releasing the thumb and forefinger. Edema of the dependent parts persisted until death.

Microscopic findings

During the first week of the deficiency there were no epidermal changes. The initial cutaneous change consisted of edema in the lower half of the cutis; the blood vessels in the edematous region were dilated and hyperemic, and there was mild, diffuse cellular infiltration throughout the cutis. In the second week there was a slight amount of loosely lamellated hyperkeratosis. Intraepidermal vesiculation was the first evidence of epidermal change. In the cutis there was cellular infiltration. During some stages the predominating cell was a mononuclear one with non-granular cytoplasm that stained diffusely with eosin. There was hyperemia in the upper as well as the lower portion of the cutis (fig. G). In some sections the edema persisted, while in other sections it subsided somewhat. In the areas of edema there was dissociation of connective tissue. In some regions there were patchy acanthosis and an increase in intraepidermal vesiculation. The edema of the cutis persisted in many sections. The extent of the cellular infiltration increased in the upper cutis, and exudative cells encroached upon the epidermis. In some sections there was a perifollicular localization of the cellular infiltration. Hyperemia was of variable extent. In the period from the fourth to the sixth week there were more epidermal changes; many of the individual cells in the several layers were vacuolated and the nuclei were shrunken. There was extrusion of keratohyalin granules into the stratum corneum and in some sections there were areas of parakeratosis and crusting. In a few areas there were small ulcers. During the period from the seventh to the fifteenth week, depending upon the area of skin examined, there was variation in the extent of cutaneous alteration, ranging from a barely discernible amount of intraepidermal edema to extensive vesiculation, with and

without crusting, to ulceration. Small and large sections of acanthotic epidermis were punctuated by areas of apparently normal epidermis. Hypermia and edema persisted in many of the sections of the paws. However, there was subsidence of the edema in many other areas, and the cellular infiltration was less dense, except in the cutis beneath those portions of the epidermis in which there was vesiculation. After the fifteenth to the twentieth week the epidermis was of normal thickness, except in acanthotic and eczematous portions. However, the thickness of the cutis often was increased considerably. There were no calcified plaques or sclerodermatous changes. Van Kossa stains revealed a small amount of calcium in the epidermis but none in the cutis, and elastic tissue stains demonstrated no decrease in, or disturbance of, elastic tissue. Throughout the disease the sebaceous glands and rudimentary coil glands were not affected except in ulcerated areas.

In the kidneys extensive degeneration of the tubules and the glomeruli and deposition of calcareous material in the areas of degeneration were observed. In the livers of many of the animals there were hyperemia, perivascular edema and occasionally disintegration of the liver cells.

Chemical findings

Moisture. Watchorn and McCance ('37) studied the moisture content of the skins of 90-day-old rats suffering with chronic magnesium deficiency. They reported that the moisture content of the skin of the deficient rats was 57.9% compared to 55.8% in normal rats. Fawns and Jung ('33) found that the moisture content of normal rat skin varied between 42.7 and 56.8%. It was considered desirable to ascertain the percentage of moisture in acute magnesium deficiency, with which we were dealing in this study, and to determine whether the alternating stages of erythema and pallor influenced the amounts of moisture. The entire body was closely shaved with small animal clippers. The skin was immediately excised, stripped of loose subcutanea and promptly weighed. The tissue was then dried for 48 hours at 105°–110° in order to obtain a constant weight. The moisture of the skins of the deficient rats in this experiment (table 1) varied from 29.1 to 41.7%; in the control group the moisture content varied from 25.13% to 47.9%. There were no significant differences in the two stages, namely erythema and pallor, of acute magnesium deficiency. The increase in moisture reported by Watchorn and McCance was probably a manifestation of the chronic stage of the deficiency and the difference between the percentage of moisture content in the rats in this ex-

TABLE 1

Showing the moisture and ash, magnesium and calcium contents of the skins of magnesium-deficient rats reared on experimental rations supplemented with adequate and inadequate amounts of the vitamin B complex.

		NUMBER OF RAT	WEIGHT IN GRAMS	AGE IN DAYS	MOISTURE		ASH [1]		MAGNESIUM [2]		CALCIUM [4]		CA. MG. RATIO
					Deficient	Control	Deficient	Control	Deficient	Control	Deficient	Control	
					%	%	%	%	*mg./100 gm.*		*mg./100 gm.*		
Crystalline vitamin B supplements equivalent to a 10% level of yeast	Stage of Erythema	M1	68	35	34.9		52.0		20.38		67.99		3.3
		N1	65	35		38.7		61.9		21.33		42.28	1.9
		M2	79	35	34.0		50.1		16.33		20.23		1.2
		N2	79	35		34.5		51.1		18.59		26.72	1.4
		M3	62	35	32.1		45.9		17.45		18.10		1.1
		N3	103	35		43.2		74.8		23.80		20.28	0.85
		M4	83	35	34.3		50.8		18.78		12.67		0.67
		N4	92	35		47.9		71.9		21.40		14.08	0.66
	Stage of Pallor	M5	85	49	33.9		52.4		12.90		13.68		1.1
		N5	87	49		26.7		50.9		16.70		16.92	1.0
		M6	118	49	41.7		70.5		23.70		15.31		0.65
		N6	91	49		32.9		47.8		15.49		...	...
		M7	78	49	29.1		39.8		14.52		12.43		0.85
		N7	78	49		26.3		35.4		16.74		12.18	0.73
		M8	60	49	33.8		49.8		18.45		12.00		0.65
		N8	85	49		47.4		60.2		17.57		8.77	0.50
Crystalline vitamin B supplements equivalent to a 1% level of yeast	Stage of Erythema	X1	76	35	45.5		40.3		22.45		20.88		0.93
		Y1	67	35		31.7		46.1		20.80		17.84	0.86
		X2	83	35	32.0		59.2		15.81		13.96		0.88
		Y2	67	35		35.7		54.3		20.33		17.59	0.87
		X3	61	35	37.7		45.6		21.39		22.31		1.0
		Y3	79	35		41.3		69.4		17.46		4.62	0.27
		X4	72	35	34.3		50.7		13.46		14.82		1.1
		Y4	84	35		29.1		39.8		16.79		8.99	0.54
	Stage of Pallor	X5	86	49	37.4		58.6		12.97		15.70		1.2
		Y6	74	49		42.8		73.8		17.89		16.71	0.93
		X6	90	49	33.3		48.7		13.62		11.74		0.86
		Y6	83	49		38.9		64.2		16.14		16.18	1.0
		X7	92	49	33.3		48.9		12.53		14.44		1.2
		Y7	57	49		31.8		45.6		16.67		19.72	1.2
		X8	81	49	31.3		44.5		12.12		9.82		0.81
		Y8	75	49		34.6		51.1		18.95		12.32	0.65

[1] Calculated on a dry basis.

[2] Methods of Analyses of the Association of Official and Agricultural Chemists, 4th Ed. 1935, p. 122.

[3] Denis, W. 1922 The determination of magnesium in blood plasma and serum. J. Biol. Chem., vol. 52, p. 411.
Fiske, S. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

[4] Greenberg, D. M., and C. E. Larson 1938 The analysis of calcium in blood and other biological material by titration with ceric sulfate. J. Biol. Chem., vol. 123, p. 191.

periment and those of Fawns and Jung ('33) and Watchorn and McCance ('37) may be due to the fact that the fat stores of the skin in the acute stage were not depleted.

Ash content. Two litters of 4-week-old rats were carefully matched and divided into groups M and N (table 1). One week later half of each group was sacrificed for analyses and 3 weeks later the other half was sacrificed for analyses (table 1). The results showed that the ash content of the deficient rats (M 1, 2, 3 and 4) varied from 45.9 to 52%; the ash content of the control rats (N 1, 2, 3 and 4) varied from 51.1% to 74.8%. This apparent difference in the ash content of groups M and N is not due to the slight differences in the magnesium content.

Magnesium content of the skin. The magnesium content of normal rat skin has been determined by Watchorn and McCance ('37). In normal rats, 90 to 120 days old, they found 29.6 mg. per 100 gm., as calculated on the basis of dry weight, and in magnesium-deficient rats of the same age 24.9 mg. Tufts and Greenberg ('38) reported that the magnesium content of the skins varied from 9.9 to 21.8 (mean 15.1) mg. per 100 gm. of fresh tissue. Their figures are not comparable to those in table 1, inasmuch as the values in this experiment are expressed in milligrams per 100 gm. of dry tissue. In their control group Tufts and Greenberg found that the magnesium content of the skin varied from 10 to 20.3 (mean 15.1) mg. per 100 gm. of fresh tissue.

In table 1 are summarized the magnesium and calcium values for magnesium-deficient rats 1 and 3 weeks after the institution of the "uncomplicated" magnesium-deficient diet. These figures represent the values for the acute stage of the disease when the rats were 35 and 49 days old. The values reported by Watchorn and McCance represent findings in the later stage. In the normal group (N 1, 2, 3, 4, 5, 6, 7 and 8) the magnesium content varied from 18.59 to 23.80 mg. per 100 gm. at 35 days, and from 15.49 to 17.57 mg. per 100 gm. at 49 days. In the deficient group (M 1, 2, 3, 4, 5, 6, 7 and 8) the magnesium content varied from 16.33 to 20.38 mg. per 100 gm. at 35 days (stage of erythema) and from 12.90 to 23.70 mg. per 100 gm. at 49 days (stage of pallor). These findings showed that the magnesium content of the skin of normal and deficient rats was somewhat lower at 35 and 49 days of age than at 90 to 120 days of age (Watchorn and McCance, '37). However, there was slight, if any, significant reduction of the magnesium content in the magnesium-deficient animals as compared with their litter-mate controls. There was no significant difference in the stages of erythema and pallor.

[3] Methods of Analyses of the Association of Official and Agricultural Chemists, 4th Ed. 1935, p. 125.
[2] Denis, W. 1922 The determination of magnesium in blood plasma and serum. J. Biol. Chem., vol. 52, p. 411.
Fiske, S. H., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.
[4] Greenberg, D. M., and C. E. Larson 1938 The analysis of calcium in blood and other biological material by titration with ceric sulfate. J. Biol. Chem., vol. 123, p. 191.

Calcium content of the skin. The calcium values showed no consistent rises in the stage of erythema. However, the highest values for calcium were during this stage. The calcium-magnesium ratios failed to furnish the anticipated information that calcium values in the skin would be increased simultaneously with a decrease in magnesium.

Differential diagnosis

The dermatoses resulting from deficiencies of the entire vitamin B complex other than crystalline thiamine (Sullivan and Nicholls, '40b), riboflavin (Sullivan and Nicholls, '41), pantothenic acid (Sullivan and Nicholls, '42b) and biotin (Sullivan and Nicholls, '42a) bear no gross or microscopic resemblances to the signs produced by deficiency of magnesium. Pyridoxine deficiency (Antopol and Unna, '39; Sullivan and Nicholls, '40a) is the only nutritional dermatoses that need be differentiated, because the involvement of the peripherae and the reported convulsive seizures (Chick, El Sadr, and Worden, '40) in pyridoxine deficiency may be sources of confusion during certain stages of the diseases. There is also some similarity in the weight curves of pyridoxine-deficient rats and magnesium-deficient rats. However, there are gross and microscopic features which distinguish the two diseases. In pyridoxine deficiency (fig. K) desquamation of the skin of the peripherae was characteristic while in magnesium deficiency (fig. J) erythema and edema of the paws were not accompanied, preceded, or followed by scaling. In pyridoxine deficiency the normal contour of the ear was distorted; the ears were shrivelled and shrunken (figs. D, E and H). In magnesium deficiency the ears were thickened and crusts and scales formed on the margins but the contour was not destroyed (figs. B, C and G). Microscopically there are distinguishing features in the cutaneous alterations. In pyridoxine deficiency there was hyperkeratosis and a peculiar type of acanthosis (fig. H). In magnesium deficiency (fig. G) there were extensive edema and hyperemia in the lower cutis, a diffuse cellular infiltration throughout the cutis and an increase in the thickness of the cutis. Chick, El Sadr, and Worden ('40) reported convulsive seizures in rats maintained for long periods, 4 to 5 months and over, on a purified synthetic diet deficient in pyridoxine. The seizures were similar in many respects to those observed in the magnesium-deficient rats, except that there was flaccidity rather than rigidity in the initial stage of the seizures of the pyridoxine-deficient rats. Also the long period of the experiment, 4 to 5 months, which preceded the onset of the convulsions in the rats of Chick and asso-

ciates was at variance with the short period, less than a week, which preceded the onset of convulsions in the magnesium-deficient rats. The extensive kidney changes and edema of the dependent parts which were characteristic of magnesium deficiency were never observed in pyridoxine deficiency.

EXPERIMENT II

THE LACK OF INFLUENCE OF THE VITAMIN B COMPLEX ON THE SIGNS OF MAGNESIUM DEFICIENCY

Greenberg and Tufts ('38) presented evidence purporting to show that the magnesium deficiency syndrome produced in rats by Kruse, Orent and McCollum ('32) was complicated by vitamin B complex deficiency. Watchorn and McCance ('37) stated that some of their magnesium-deficient animals showed signs of vitamin B complex deficiency although the controls were normal. This suggested that the requirements of the vitamin B complex were higher or that vitamins of the B complex functioned less efficiently when the magnesium level was low. In order to examine the possible relationship of the vitamin B complex and magnesium deficiency, the signs and symptoms produced by several variations of the magnesium-deficient diet were evaluated.

A. A comparison of magnesium deficiency signs produced by diets containing crystalline vitamin B complex supplements of high and low levels

The signs of magnesium deficiency were studied in two groups of carefully paired young litter mates. To one group was fed the diet and supplement described in experiment I; the amount of vitamin B complex therefore corresponded to a 10% level of a potent yeast. To the other group was fed the same diet with the exception that the crystalline B vitamins were incorporated at a level that corresponded to the amount of the B vitamin in 1% yeast. The onset and severity of the cutaneous signs of magnesium deficiency were essentially the same for each group. Growth was inferior in the latter group. The convulsions were not delayed in the latter group but occurred at the same period (within 2 days) of the convulsions in the group that was adequately supplemented with the vitamin B complex. In three of the litters of the first group there developed in the late stage certain non-specific, cutaneous signs which included mild, generalized, brown scaling on the back, dishevelled fur and alopecia on the venter. These signs vaguely suggested superimposed riboflavin deficiency. Adminis-

tration of large doses of riboflavin and the entire vitamin B complex supplement had no effect on the signs.

Chemical findings

Sixteen 28-day-old rats, that were the litter mates of the rats in groups M and N of experiment I, were evenly matched and divided into two additional groups, X and Y (table 1). To group X was fed a magnesium-deficient diet containing crystalline vitamin B complex supplements, incorporated at a level corresponding to 1% yeast. To group Y was fed a magnesium control diet also containing crystalline vitamin B complex supplements, incorporated at a level corresponding to 1% yeast. The moisture content, the percentage of ash, the amount of magnesium and calcium per 100 gm. of dry tissue, and the calcium-magnesium ratios of the skin of half of each group were determined 1 week later during the stage of erythema and 3 weeks later during the stage of pallor. These results were compared with the values for groups M and N, that were supplemented with an adequate amount of vitamin B complex. The data indicated that the level of the vitamin B complex was unimportant insofar as these values were concerned. In the vitamin B complex-deficient magnesium-deficient rats the magnesium content at 35 days of age was approximately equal to that of the control groups (N and Y) that were supplemented with adequate and inadequate vitamin B complex. However, at 49 days the magnesium content of the skins of the vitamin B complex, magnesium-deficient rats was slightly lower than that of their controls and of groups M and N.

B. A comparison of the therapeutic response of vitamin B complex deficiency other than thiamine hydrochloride to treatment with magnesium-deficient diets containing high and low levels of the vitamin B complex

Three groups of young animals were fed a diet deficient in the entire vitamin B complex except crystalline thiamine hydrochloride. The animals lost weight. One month later when they were practically in extremis, and therefore could be considered to have been well depleted of the vitamin B complex other than thiamine, the original ration was discontinued. Microscopic examination showed atrophy of the epidermis, the sebaceous glands and the cutis. The rats were then divided into two groups to which magnesium-deficient diets containing inadequate and adequate amounts of the vitamin B complex were fed.

To one group, the vitamin B complex was supplied as crystalline factors incorporated at a level equivalent to 1% yeast of known potency. To the other group the vitamin B complex was incorporated as crystalline factors in amounts equivalent to the vitamin B complex contained in 10% yeast. All of the animals recovered from their almost moribund state. The rats in the group that received the vitamin B complex, equivalent to 1% yeast, gained 6 gm. during the first month after the magnesium-deficient diet was started; the rats in the group that received the vitamin B complex equivalent to 10% yeast gained 29 gm. during the month after the magnesium-deficient diet was started. The onset of hyperemia and hyper-irritability and the convulsive seizures occurred within 3 to 4 days in the two groups, and the duration and severity of the cutaneous signs were identical. On microscopic examination it was found that the epidermis and cutis were no longer atrophic and that there was partial to complete restitution of the previously atrophic sebaceous glands; in the lower cutis there were edema and hyperemia and throughout the cutis there was a cellular infiltration characteristic of magnesium deficiency. These findings demonstrated that the addition of adequate and inadequate amounts of the crystalline supplements of the vitamin B complex resulted in a cure of the signs due to deficiency of the vitamin B complex other than thiamine, in skin manifesting histologic evidence of magnesium deficiency.

C. The evaluation of magnesium deficiency signs in young rats reared on a diet containing a commercial liver extract known to be low in magnesium and pyridoxine

The signs of magnesium deficiency were evaluated in ten young rats reared on a magnesium-deficient diet in which a commercial liver extract [5] known to be low in magnesium was incorporated at a 1.5% level as the source of the vitamin B complex. The levels of the individual components of the vitamin B complex were considerably less than those in experiment I. The time of the onset and duration of the signs corresponded to those of experiment I. In another litter of rats the amount of the commercial liver extract was doubled and the onset, duration and severity of the signs were not influenced. The amount of pyridoxine in this source of the vitamin B complex is com-

[5] Lederle's Vitamin B Complex. Each ml. contains approximately 375 μg. of thiamine hydrochloride. 150 μg. of riboflavin and 50 μg. of pyridoxine. When incorporated at a 1.5% level the amount of pyridoxine was approximately one-twelfth that which the rats in experiment I received. When the amount of the extract was doubled the pyridoxine content was still comparatively low.

paratively low; therefore additional daily supplements of 500 μg. of crystalline pyridoxine were administered to two rats. The additional pyridoxine had no influence on the signs.

D. The lack of influence of massive doses of pyridoxine on the signs of magnesium deficiency

To two litters of 21-day-old rats the diet described in experiment I was fed. The supplements of pyridoxine were increased five-fold, i. e., from 10 mg. to 50 mg. Again there was no influence on the cutaneous signs, the irritability and the convulsive seizures.

E. Lack of influence of massive doses of thiamine, riboflavin, pyridoxine, pantothenic acid, para-amino-benzoic acid, nicotinic acid and choline on the signs of magnesium deficiency

In order to complete the observations on the possible influence of the vitamin B complex on the signs of magnesium deficiency, the crystalline vitamin B complex supplements listed above were incorporated into the diet at a level five times as great as that described in experiment I. This had no influence on the onset or severity of the cutaneous signs, the hyperirritability and the convulsive seizures.

EXPERIMENT III

LACK OF THE INFLUENCE OF VITAMIN E ON MAGNESIUM DEFICIENCY SIGNS

The supplement of alpha-tocopherol used throughout these experiments was omitted from the rations of five rats in a litter of eight. There was no influence on the onset, duration and severity of the signs.

SUMMARY AND DISCUSSION

The signs resulting from magnesium deprivation were described in 1931–32 by McCollum and Orent, and Kruse, Orent and McCollum. These investigators produced a symptom-complex comprising vasodilatation, hyperirritability of the nervous system, cardiac arrhythmia, spasticity and tonic-clonic convulsions. The experimental diet included only 1.8 parts per million of magnesium, but was considered otherwise adequate. A yeast extract corresponding to approximately a 13.5% level was incorporated into the ration. Assuming that the yeast was a potent source of the vitamin B complex, the diet was probably adequate in all respects, except that it lacked magnesium and vitamin E. Inas-

much as the signs occurred within 1 to 3 weeks, it is likely that the inclusion of vitamin E would not have affected the signs. In 1938, Greenberg and Tufts studied the influence of several factors on the incidence, time of onset, and duration of the peripheral vasodilatation and hyperirritability in magnesium-deficient rats. They stated that the signs were affected by the level of the vitamin B complex, and criticized the use of the alcoholic extract of yeast employed by Kruse, Orent and McCollum, because it was apparently low in factors of the vitamin B complex. Of the several deficiency syndromes produced by deficiencies of the components of the vitamin B complex, pyridoxine deficiency is the only one likely to be confused with magnesium deficiency, because of the apparent similarity of the involvement of the peripherae and the reported convulsive seizures in pyridoxine deficiency (Chick, El Sadr and Worden, '40). The findings in experiment I demonstrated that: (a) the gross signs and symptoms of magnesium deficiency may be differentiated readily from those of pyridoxine deficiency; and (b) the histologic findings in the two diseases are dissimilar. The findings in experiment II demonstrated that: (a) the level of the vitamin B complex exerted no influence on the onset and/or severity of the cutaneous signs and the convulsive seizures, and (b) magnesium deficiency signs in the skin could be produced simultaneously with the healing of the signs of deficiency of the vitamin B complex. The data in experiment II also showed that the magnesium content of the skin is similar in rats subsisting on diets containing adequate and inadequate vitamin B complex.

In acute magnesium deficiency the percentage of magnesium of the skin was found to be only slightly lower than the percentage in normal animals. Likewise, the moisture and ash contents and the level of calcium were not significantly altered. When the chemical findings of the magnesium-deficient, vitamin B complex-deficient rats were compared with those of the magnesium-deficient rats that were adequately supplied with the vitamin B complex, the data showed that there was no appreciable influence of the vitamin B complex on the chemical findings.

In experiment III it was demonstrated that vitamin E was unrelated to the signs of magnesium deficiency.

CONCLUSION

The data in these experiments show that there is no significant interrelationship of magnesium deficiency and the deficiencies of the vitamin B complex.

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PLATE

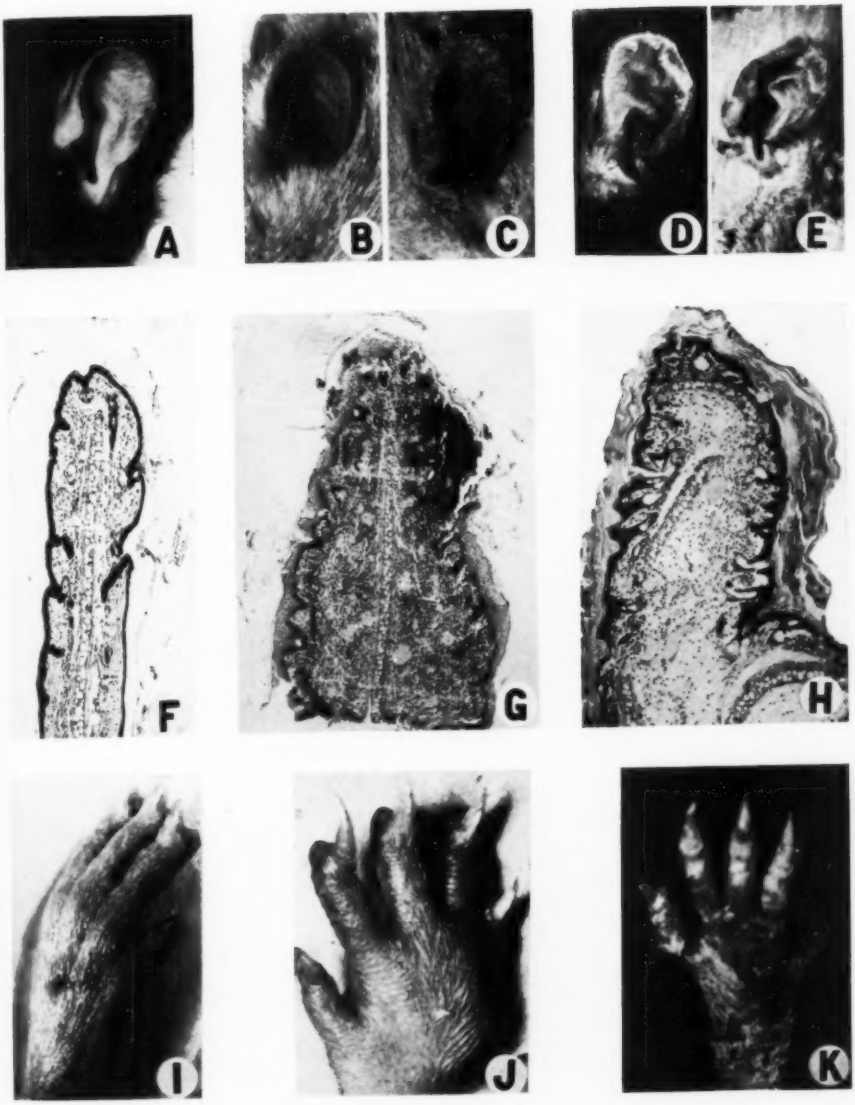
PLATE 1

EXPLANATION OF FIGURES

In the left-hand column at A, F and I are portrayed a normal ear and a normal foot. In the middle column are portrayed at B, C, G and J signs of magnesium deficiency. In the right-hand column at D, E, H and K are signs of pyridoxine deficiency.

In the early stage of magnesium deficiency (B) the ear is erythematous and swollen; the contour of the ear is not disturbed. In the late stage of magnesium deficiency (C) the ears are thickened and on the margins there is crusting and scaling. In the early stage of pyridoxine deficiency (E) the ear is shrivelled and shrunken. Microscopic changes of the ear in magnesium deficiency (G) consist of edema, diffuse cellular infiltration throughout the cutis, hyperemia, acanthosis alternating with apparently normal epidermis, and slight if any hyperkeratosis. On the tip of the ear there is ulceration and crust formation. In pyridoxine deficiency the cartilage is distorted, there is only a slight amount of cellular infiltration in the cutis, the acanthosis is of a different type and there is extensive hyperkeratosis.

In magnesium deficiency the paws (J) are swollen and erythematous. Desquamation does not precede, accompany or follow the erythematous stage. In pyridoxine deficiency (K) scaling of the dorsa of the paws is a characteristic feature.


A
B
C
D
E
F
G
H
I
J
K

NUTRITION AND TOLERANCE TO ATABRINE[1]

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(Received for publication, September 8, 1943)

Little study has been made of the effect of continuous drug administration upon the nutritive requirements, or conversely, of the effect of nutritive state upon the ability of the animal to withstand pharmacological agents. However, a survey of the literature reveals sufficient evidence to indicate that the action of some drugs may depend to a certain extent upon the diet of the animal. The ability to raise animals as the rat, chick, and dog, on diets of more or less known constitution affords a tool for the investigation of the relation between nutrition and pharmacology.

Perhaps the best known example of such a relationship is that of certain sulfonamides. Black, McKibbin and Elvehjem ('41) showed that on a purified diet supplemented with the crystalline vitamins the inclusion of sulfaguanidine in the diet depressed the rate of growth of young rats. This effect could be prevented by feeding liver extract and partially overcome by para-aminobenzoic acid. Similar and more extensive work of various laboratories has been reviewed (Anonymous, '43a, '43b). Deficiencies of biotin, folic acid and vitamin K have been reported in rats receiving sulfaguanidine or succinylsulfathiazole. It seems apparent that these nutrients, on the experimental diets used, were supplied by an intestinal flora which became inadequate in the presence of the sulfonamides. Kornberg, Daft and Sebrell ('43) have shown that the granulocytopenia and anemia produced in rats by feeding sulfonamides can be cured by liver extracts. Xanthopterin (Totter and Day, '43) may be one of the factors involved. Daft and coworkers (Daft, et al., '43) have presented evidence that muscle lesions caused by feeding sulfasuxidine are actually an alpha-tocopherol deficiency.

Less evidence is available on the effect of diet upon the therapeutic action of the sulfonamides. However, McCarty ('41), Selbie ('40),

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

Maier and Riley ('42), Findlay ('40) and others have shown that p-aminobenzoic acid will abolish the therapeutic action of these agents in several diseases.

The toxicity of several other agents has also been related to diet. The carcinogenicity of butter yellow apparently depends upon protein (Kensler et al., '41) or choline and cystine (Gyorgy et al., '41) and biotin (du Vigneaud et al., '42). Methionine and cystine prevent growth inhibition of butter yellow on low protein diets (White, '40). High protein diets also decrease the toxicity of trinitrotoluene (Himsworth and Glynn, '42) and of selenium (Smith and Stahlman, '40; Anderson et al., '41).

The detoxification of certain arsenicals by para-aminobenzoic acid has been demonstrated by Sandground and Hamilton ('43a, '43b). It may be important from the therapeutic standpoint that the para-aminobenzoic acid did not inhibit the trypanocidal action of the arsenicals, although their toxicity was much decreased.

The antimalarial drugs, atabrine and quinine, are administered prophylactically and in suppressive treatment over long periods of time. Because malaria is endemic in so many regions and particularly because certain currently strategic areas are highly infested with malaria, any knowledge relating diet to tolerance of antimalarials is opportune. This paper reports experiments designed to determine whether various dietary regimens affect the toxicity of atabrine over extended periods.

EXPERIMENTAL[1]

All of the studies on rats were made with young albino rats weighing 40 to 60 gm. purchased from the Rockland Farms. They were housed in individual cages, and groups of 5 or 6 rats were fed each experimental ration ad libitum except where otherwise noted.

The experimental rations used in most of the studies were as follows: sucrose 74%; casein[2] 18%; salt mixture 4% (Hegsted et al., '41); and corn oil 4%. Vitamins were added at the following levels per 100 gm. of ration: thiamine chloride 100 μg., riboflavin 200 μg., pyridoxine 100 μg., calcium pantothenate 1.6 mg., nicotinic acid 1.0 mg., and choline 150 mg. Vitamins A, D and E were fed by dropper twice weekly to supply 80 I. U. of A, 1.5 I. U. of D, and 0.5 mg. of alpha-

[1] We are indebted to Merck & Company, Rahway, New Jersey, for furnishing the crystalline B-vitamins and the alpha-tocopherol, and to Abbott Laboratories, North Chicago, Illinois, for furnishing Haliver Oil.

[2] S. M. A.

tocopherol per rat per week. The above levels of the water soluble vitamins were chosen for the original experiments to be near or slightly below the optimum level since it was thought that if the requirement of any of these was increased by atabrine it would be detected at these levels. In later experiments, when a single vitamin was investigated, it was simply omitted from the diet and the levels of the other vitamins doubled, except choline, which was fed at 100 mg. per 100 gm. of ration. Atabrine dihydrochloride was added to the appropriate diets by dissolving in alcohol and drying on the ration.

The general plan for the study of the vitamins which were investigated was to deplete two large groups of animals, one of which received atabrine (usually 40 mg. per 100 gm. of ration) in the diet. When the animals were depleted (symptoms or failure to gain), they were divided into groups and fed graded levels of the vitamin in supplement cups or by dropper. The gains of the animals with and without atabrine and receiving the same level of supplement were compared.

In studies with chicks, day-old White Leghorn cockerels were used; they were housed in groups of six in heated cages and fed the appropriate ration and water ad libitum.

RESULTS

General

Atabrine has been fed to rats at levels from 0 to 65 mg. per 100 gm. of ration. Food consumption records of these young animals showed that they consumed an amount equal to approximately 10% of their body weight per day. Thus atabrine levels per 100 gm. of ration in short experiments are similar to levels in terms of per kilogram of body weight per day. Over a 6 months' period atabrine levels of 25 mg. % or less caused no growth retardation or other abnormalities. Over a 3 months' period levels of 40 and 65 mg. % prevented normal growth (20 to 35% less) but except for yellowing of the skin and a ruffled coat, no other abnormalities were noted. Actual atabrine intake of these animals is unknown but is somewhat less than suggested, as rats of this age consume less food relative to body weight.

Rather complete histological studies were made of a few rats which had received atabrine at levels of 25 and 40 mg. % for 6 months. Only minimum micropathologic changes were noted. These consisted of the appearance of granules in the Kupffer cells of the liver, the glomerular epithelium and occasionally the tubular epithelium of the kidney, in the spleen and in heart muscle. These granules are presumably ata-

brine. In teased-out preparations of kidney placed in strong potassium iodide solution the granules are very conspicuous and decidedly brownish in color. The early storage of atabrine or an atabrine derivative in the epithelial cells covering the glomerular capillaries is of interest. No necrotic changes were detected.[4]

A diet containing all of the vitamins at five times the levels given above and another containing brewers' yeast at a level of 4% were fed with and without 40 mg. % of atabrine. Although the growth was somewhat improved, the atabrine groups still gained at approximately 25% less than the control. Apparently growth inhibition is not due to lack of intestinal synthesis of nutrients furnished by yeast nor can it be overcome by high levels of the known vitamins.

TABLE 1

Average gain in weight on various levels of riboflavin with and without atabrine in the diet.

MICROGRAMS OF RIBOFLAVIN PER DAY	TIME OF EXPERIMENT	NO ATABRINE	PLUS ATABRINE	PLUS ATABRINE AS % OF CONTROLS
Experiment I	*days*	*grams gain*	*grams gain*	
0	25	11.5	11.5	100
6	25	48.0	35.5	74
12	25	...	40.5	...
30	25	...	55.5	...
Experiment II				
4	43	55.5	42.6	77
8	43	80.0	71.8	89
12	43	99.0	81.5	82

Riboflavin deficiency

Thirty-six male rats were depleted of riboflavin, twenty-four of which were on the diet containing 40 mg. % of atabrine. After 57 days the controls were divided into two groups which received 0 and 6 μg. of riboflavin per day, and the atabrine rats into four groups which received 0, 6, 12, and 30 μg. of riboflavin per day. A comparison of the two groups receiving 6 μg. of riboflavin (table 1) shows that, as on a complete diet, the atabrine rats gained about 25% less than the controls. Over 12 μg., but less than 30 μg., of riboflavin per day were required before the atabrine groups equaled the gain of the controls. No difference was noted in the two groups which received no riboflavin.

[4] The pathologic examinations, including preparation of the sections, were done by Dr. S. B. Wolbach, Harvard Medical School, for which the authors are deeply grateful.

In a confirmatory experiment the depletion period was only 35 days and riboflavin was fed at 4, 8 and 12 μg. per day to groups both with and without atabrine. Similar but less marked differences were obtained (table 1). It appears that the long depletion period with atabrine in the first experiment had limited the ability of the animals to respond.

Protein deficiency

Diets containing 10, 20, and 40% of casein were fed with and without atabrine at a level of 40 mg. %. Over a period of 6 weeks the animals receiving atabrine gained 27, 15, and 19% less than the corresponding control animals. The results appear to be similar to those obtained with animals on riboflavin deficient and complete diets.

Vitamin A deficiency

Thirty-six animals, half of which received atabrine (40 mg. %), were depleted of vitamin A until eye symptoms were noted and the animals began to lose weight (24 days). Groups with and without atabrine were then given daily supplements of 1, 3 and 5 μg. of carotene

TABLE 2

Average gain in weight on various levels of carotene with and without atabrine in the diet.

MICROGRAMS OF CAROTENE PER DAY	TIME OF EXPERIMENT	NO ATABRINE	PLUS ATABRINE	PLUS ATABRINE AS % OF CONTROLS
	days	*grams gain*	*grams gain*	
1	31	15.7	16.1	102
3	39	54.0	58.0	107
5	39	77.4	70.8	91.5

dissolved in corn oil. The gain in weight of the six groups after supplements were started is shown in table 2. There appears to be no growth inhibition due to atabrine except at the highest level of carotene, which is in contrast to the studies on riboflavin and protein.

Reproduction

Three males and three females which had receivd the basal diet plus 25 mg. % of atabrine from weaning until they were 5 months of age were transferred to a diet of ground commercial dog food [5] to which was added 25 mg. % of atabrine. They were mated when 6 months

[5] Purina Dog Chow.

of age. Normal litters of 8, 10, and 6 young have been reared, are now 4 months old and show no abnormalities.

Chick experiments

The chick is much more resistant to atabrine administration than the rat. Five-day-old chicks in groups of six were fed a standard chick mash to which atabrine was added at eight levels from 0 to 240 mg. %. Approximately 160 mg. % of atabrine was required to decrease the rate of gain about 30%. Thus by this criterion the chick can tolerate about four times as much atabrine as the rat. At higher levels of atabrine no effect, other than poor growth and discoloration, were noted. A partially purified adequate diet containing casein at levels of 10, 15, and 20% was fed. As in the rat experiments, the per cent inhibition of atabrine (160 mg. %) was approximately the same at the various levels of protein.

DISCUSSION

It seems clear from these results that atabrine at the levels fed is relatively non-toxic for the rat, and much less so for the chick. In the rat the continuous feeding of a level of 25 mg. per kilogram per day produces no gross symptomatology over a period of 1 year and reproduction is apparently normal. Wright and Lillie ('43) have recently described the pathologic changes produced in rats with high levels of atabrine. The atabrine was administered by stomach tube. At lower levels of atabrine (30 mg. per kilogram, given over a period of seven weeks) little if any effect on growth was noted and only slight pathologic lesions were observed. Care must be used in relating these data to humans, however, since the difference in rats and chicks makes it clear that species vary in ability to handle this drug. Whether the human is more or less tolerant than these species is at present unknown. It probably is significant, however, that we have fed a level of atabrine near that required to inhibit growth for 1 year with no apparent ill effect.

The significance of the effects on deficient diets is not clear. On adequate diets the growth inhibition cannot be overcome by increasing the level of protein or by high levels of vitamins or by yeast. On riboflavin-low or protein-low diets, when the animals are growing slowly, the addition of atabrine decreases the rate of growth still further. Thus they appear to react in the same manner as well nourished animals. In partial vitamin A deficiency, however, the rate of growth

was not decreased by the same level of atabrine used in the previous experiments; it would appear that when vitamin A was the limiting factor atabrine had no effect. On the other hand, the atabrine inhibition in normal animals, or when riboflavin or protein are limiting, may be related to the metabolism of these factors. The metabolism of these is interrelated as shown by Sarett, Klein and Perlzweig ('42). A direct inhibition of riboflavin is improbable since on the riboflavin-low ration and without supplements those animals receiving atabrine did not stop growing or show other signs of deficiency earlier than the controls.

The relatively short depletion period that is required to produce vitamin A deficiency as compared with the riboflavin depletion period may be of importance in the response obtained since less marked effects were observed in the second of two riboflavin experiments when the depletion period was less. However, animals on an adequate diet show a growth inhibition within the time of the depletion period, and a marked growth inhibition over a period as long as the total vitamin A experiment. Preliminary experiments indicate that the maximum level of atabrine in the tissues is reached after a period of from 3 to 4 weeks on a diet containing 40 mg. % atabrine.

CONCLUSIONS

1. Atabrine added to an adequate diet in levels such that the rat receives 25 mg. per kilogram per body weight per day or less is completely non-toxic as judged by growth, general appearance and behavior, gross and micropathology, and reproductive ability.

2. Atabrine levels of 40 to 65 mg. per 100 gm. of ration retarded growth by 20 to 30%; the fur is discolored, and the animals are unkempt. The addition of various vitamins, yeast or protein to an already adequate diet does not prevent these changes.

3. The slow growth obtained on suboptimal levels of riboflavin or protein is further decreased by the addition of 40 mg. % atabrine to the diet. With diets suboptimal in vitamin A addition of atabrine does not cause a further reduction in growth rate.

4. On the basis of rate of growth on diets containing atabrine, the chick is from three to four times as tolerant of atabrine as is the rat.

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THE EFFECT OF ATABRINE ON CHOLINE DEFICIENCY IN THE YOUNG RAT [1]

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ONE FIGURE

(Received for publication September 8, 1943)

Investigations designed to study the effect of various dietary constituents upon the tolerance of rats to the continued administraion of the anti-malarial drug atabrine have been reported (Hegsted, McKibbin and Stare, '43). As a part of these studies a low-protein, low-choline diet was fed to weanling rats, with and without atabrine at a level of 40 mg. per 100 gm. of ration. Four of five rats on the control diet died, whereas, to our surprise, none of those receiving atabrine died. This effect of atabrine has been further investigated and is the basis of this paper.

EXPERIMENTAL [2]

In all experiments weanling male rats purchased from the Rockland Farms have been used. These were placed immediately upon the experimental ration which was fed ad libitum except in one experiment where the food intake of the control group was limited to that consumed by those receiving atabrine. Atabrine was incorporated in the rations by dissolving the dihydrochloride in alcohol and drying on the ration.

Two different diets have been used. The first was low in protein and choline and had the following percentage composition: casein (SMA) 10, lard 20, salt mixture 4 (Hegsted et al., '41), and sucrose 66. Vitamins were added to supply 200 μg. thiamine chloride, 400 μg. riboflavin, 200 μg. pyridoxine, 1.5 mg. calcium pantothenate, 2.5 mg. nicotinic acid per 100 gm. of ration. The fat soluble vitamins were fed by

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] We are indebted to Merck & Company, Rahway, New Jersey, for furnishing the crystalline B-vitamins and the alpha-tocopherol, and to Abbott Laboratories, North Chicago, Illinois, for furnishing Haliver Oil.

dropper in corn oil to supply 80 I. U. of vitamin A, 1.5 I. U. vitamin D, and 1.0 mg. of alpha-tocopherol per rat per week. The second diet was similar to that used by Engel and Salmon ('41) in studies on choline deficiency. It contained 30% alcohol extracted peanut meal, 6% SMA casein, 6% corn oil, 4% salt mixture (Hegsted et al., '41), and 0.5% K_2HPO_4. Water soluble and fat soluble vitamins were administered as in the other diet and at the same levels.

RESULTS

In the first experiment using the low protein diet four of the five animals on the control diet died between the tenth and fourteenth day. The animals on the same diet plus 40 mg. of atabrine per 100 gm. of ration showed a slight decrease in weight during this period but then began to gain slowly and gained 32 gm. on the average in the next 2 months. This experiment was repeated using six rats in each group with similar results. Three of the control animals died before the fourteenth day at which time all of the animals were killed and their kidneys weighed and examined for gross lesions. Only slight damage was apparent in the atabrine group and the average weight of the individual kidneys was 260 mg. compared to 353 mg. for the controls without atabrine. Practically no growth was obtained during the first 2 weeks of the experiment. The average weight of both groups at the beginning was 30.6 gm.; those in the atabrine groups weighed 32.8 gm. at the time they were killed compared to 30.7 for the controls remaining on the fourteenth day.

Practically the same results were obtained using the peanut meal ration which is adequate except for choline. Four of five animals died on this ration of choline deficiency within 10 days while no deaths occurred when atabrine was incorporated in the ration at a level of 40 mg. %. The detailed results of the second experiment with this diet are included in table 1. All of the animals were killed on the seventh day when the first animal in the control group became moribund. The kidneys were weighed and examined for gross lesions, and the liver fat determined in the dried livers by chlorform extraction. At this early date some of the animals had not yet developed gross symptoms and the differences are not as great as might be expected had the animals been continued for a few days longer. Only slight protection was noted in the group receiving atabrine at the 40 mg. % level. It seems probable, however, from the previous experiment that these animals would have survived the critical period of choline

TABLE 1

Data obtained on rats fed a low choline ration with various supplements.

SUPPLEMENT	TIME ON EXPERIMENT	AVERAGE WEIGHT OF ANIMALS	AVERAGE WEIGHT OF KIDNEYS	KIDNEY WT. AS % OF BODY WEIGHT	ANIMALS WITH HEMORRHAGIC KIDNEYS	LIVER FAT
	days	*grams*	*mg.*		*%*	*% dry wt.*
None	7	47.5	343	1.44	60	37.4
40 mg.%[1] atabrine	7	44.2	359	1.63	40	37.2
65 mg. %[1] atabrine	7	43.9	284	1.28	0 (†)	43.7
200 mg. %[1] choline	7	59.0	356	1.21	0	13.0
None [2]	12	54.0	616	2.21	83	33.4
65 mg. %[1] atabrine	12	57.3	414	1.53	33	44.5

[1] Mg. per 100 gm. of ration.

[2] Food intake limited to the amount consumed by the animals receiving 65 mg. % atabrine in the diet.

deficiency (Griffith, '41). At the higher level of atabrine the protection was practically complete although a few showed slight signs of kidney damage. Atabrine appeared to have no lipotropic action.

Since Griffith ('41) has shown that limiting the food intake will decrease the severity of kidney damage, this experiment was repeated and the food intake of the atabrine group was measured and that of the control group limited to this amount. As the control animals died their kidneys were examined and weighed, and the livers dried for fat analysis. All of the remaining animals were killed on the twelfth day. The growth curves for these groups are shown in figure 1 and the details of our analyses in table 1. Contrary to our experience with adequate diets (Hegsted, McKibbin and Stare, '43), the efficiency of

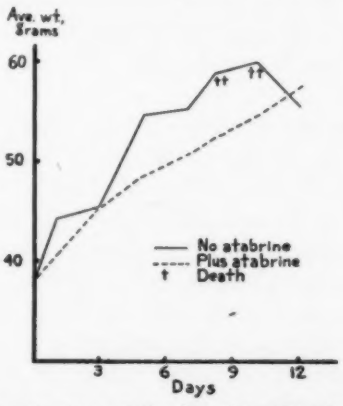

Fig. 1 Growth curves for animals on choline-low diet with and without atabrine at 65 mg. per 100 gm. of ration.

food utilization was somewhat impaired by atabrine administration. Four of the five animals on the control diet showed severe kidney damage and the kidney weights of these four were well above 2% of the body weight. Only two of the animals receiving atabrine had gross kidney lesions and in only one of these was the kidney weight in excess of 2% of the body weight.

DISCUSSION

Moyer and du Vigneaud ('42) have summarized the data upon those compounds which have a choline-like action in the chick and rat. The action of all such compounds reported to date can be explained upon the basis of transferable methyl groups or by the replacement of choline directly (arsenocholine, triethylcholine). The action of dimethylaminoazobenzene as a methyl donor has been reported by Jacobi and Baumann ('42) and indicates that the list of compounds with available methyl groups may be extensive indeed. The availability of methoxyl groups, the only source of methyl groups in atabrine, has not yet been reported and must await further investigation. Two N-ethyl groups are present in atabrine. Since triethylcholine prevents hemorrhagic kidneys, one might speculate on the possibility of these groups acting to replace methyl groups. However, there appears to be no evidence that transethylations occur in the body and it appears more likely that triethylcholine acts directly by replacing choline.

In their review on tissue changes in vitamin deficiencies Wolbach and Bessey ('42) mention, "Although the mechanism underlying the hemorrhages is not clear, the function of acetylcholine as a neuromuscular mediator is suggestive of a neurovascular cause." This suggests that those drugs affecting the choline esterase system might play a role in modifying the kidney lesions. This phase of the problem is being investigated.

Finally the part played by food intake and growth must be considered. By the paired feeding method our results were as clear cut as with ad libitum feeding. However, the control animals gained somewhat more on the limited food intake than the animals receiving atabrine on the peanut meal ration. On the low-protein ration the gains for the animals with and without atabrine were practically the same although the food intake was not measured. If these rather slight differences are the determining factor, a great deal of care must be exercised in studies on compounds being tested for choline-like action.

The average food intake of our rats on the peanut meal ration plus 65 mg. % atabrine was from 5 to 5.7 gm. per day over the 12 days

on experiment. This represents an atabrine intake of 3.2 to 3.7 mg. per day which is more than required to prevent death on this choline-low ration. Engel states that approximately 5 mg. of choline is required on a similar ration to allow complete survival. This indicates a high degree of activity in view of the fact that atabrine has a molecular weight approximately three times that of choline, although no direct comparison has been made on our ration.

CONCLUSIONS

The inclusion of atabrine in low choline diets at a level of 65 mg. per 100 gm. of ration almost completely prevents hemorrhagic kidneys in the rat. A level of 40 mg. per 100 gm. prevents death but allows some kidney damage to develop. No lipotropic action was observed at the levels fed.

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VITAMIN A STORAGE AND FACTORS THAT AFFECT THE LIVER[1]

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ONE FIGURE

(Received for publication August 19, 1943)

While the storage of vitamin A in the liver depends primarily upon the amount of vitamin absorbed from the digestive tract, this storage can also be modified by certain agents that affect the liver itself. Ethyl alcohol (Ikegaki, '38), thorotrast (Lasch and Roller, '36) bismuth (Wendt and Koenig, '37), and carcinogens such as 1, 2, 5, 6-dibenzanthracene (Goerner and Goerner, '39) are all reported to hasten the depletion of hepatic stores of the vitamin, and it has been suggested (Baumann, Foster and Moore, '42) that the hydrocarbon acts by interfering with a combination of vitamin A and protein or some other "anchor" in the liver. However, not all metabolic changes in the liver are accompanied by changes in hepatic vitamin A. In starvation the glycogen content of the liver diminishes without any parallel loss of the vitamin (Dann and Moore, '31). Phosphorus (Lasch, '35) is reported to produce degeneration of the liver without effect on vitamin A storage. Abels et al., ('42) concluded that the storage of vitamin A is independent of general hepatic dysfunction. The present experiments deal with the hepatic storage of vitamin A in rats or mice fed substances that in one way or another modify biochemical reactions taking place in the liver.

METHODS

All experiments were performed in series involving groups of animals comparable in weight, sex and previous vitamin A intake. Initial stores of the vitamin were established by feeding halibut liver oil. The various groups within the series were then fed diets free from vitamin A but involving other modifications. After a suitable period of depletion, usually 6 or 8 weeks, the animals were killed for analysis and the vitamin A content of the liver determined colorimetrically (Davies, '33;

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Wisconsin Alumni Research Foundation.

Baumann, Riising and Steenbock, '34). The basal diet deficient in vitamin A (diet I) consisted of heated casein 18%, salts 4%, yeast 7.9%, irradiated yeast 0.1%, agar 2%, cottonseed oil 5% and dextrinized starch 63%.

EXPERIMENTAL

p-Dimethylaminoazobenzene and hepatic storage of vitamin A

In a previous experiment colloidal suspensions of dimethylaminoazobenzene were injected intraperitoneally for 8 weeks into rats fed diet I (Baumann, Foster and Lavik, '41) with practically no effect of the dye on the vitamin although the carcinogenic hydrocarbons markedly reduced the storage of vitamin A under these conditions. Diet I, however, also tends to minimize the production of liver tumors due to p-dimethylaminoazobene (Miller et al., '41).

In the present experiments the rate of depletion of vitamin A was measured under more optimal conditions for the production of liver tumors with the azo dye (Miller et al., '41). The basal diet deficient in vitamin A was also low in riboflavin and it was not particularly high in protein (diet II). In consisted of 12% of heated casein, 2% of rice bran concentrate, 4% of salts, 5% of cottonseed oil and 77% of dextrin. Dimethylaminoazobenzene was added to the cottonseed oil as 0.06% of the diet.

Twenty rats, averaging 93 gm. in weight, were each fed 2 drops of halibut liver oil to establish moderately high stores of vitamin A. Two rats were killed 3 days later and the vitamin content of the livers found to average 502 μg. of vitamin A per rat. The remaining rats were then divided and the first group fed diet II ad libitum. A second group was fed the same diet ad libitum, but minus the azo dye (diet III), while a third group was fed diet III in the amounts consumed by the group getting the carcinogen. The effect of the dye was also determined on two other groups fed diet I with and without the carcinogen. Six weeks later all rats were killed for analysis.

No pronounced variation in vitamin A storage resulted from the feeding of the azo dye. After 6 weeks of depletion the hepatic stores averaged 374 μg. of vitamin A per liver on the carcinogenic low A diet, (diet III) as compared to 355 μg. in the presence of the azo dye (diet II). On diet I, which tends to minimize tumor formation, the figures were 386 μg. in the absence of the dye as compared to 353 μg. in its presence. These differences in averages were not considered significant. The slight lowering of hepatic vitamin A in the groups fed the dye was probably caused by a reduced consumption of food rather than by the dye itself,

since the group on the restricted food intake contained the same amounts of hepatic vitamin A as the group fed the dye. Certainly the effect of the azo dye was very much less than the effects previously observed with alcohol or with the carcinogenic hydrocarbons (Baumann et al., '41, '42). Extracts of the combined organs (lungs, kidneys, adrenals, spleen and blood) from animals receiving the dye yielded a red color with $SbCl_3$.

TABLE 1

The effect of p-Dimethylaminoazobezene on hepatic vitamin A.

DIET	AZO DYE	WEEKS ON EXPT.	AVERAGE WEIGHT OF RATS	VITAMIN A PER LIVER	NO. OF RATS
	%		gm.	μg.	
	0	0	106	502 (436–604)	2
I — ad lib.	0	6	179	386 (359–408)	3
I — ad lib.	.06	6	133	353 (346–361)	2
II — ad lib.	.06	6	94	355 (343–380)	5
III — ad lib.	0	6	129	374 (365–392)	3
III — restricted	0	6	102	354 (330–395)	3

Diet I contained 18% of casein and 8% of yeast. Liver tumors do not develop readily on this diet.

Diet II contained 12% of casein and 2% of a rice bran concentrate. On this diet the incidence of liver tumors frequently reaches 100% in 4 to 6 months. Diet III is the same as Diet II except that the dye is omitted.

3, 3′-methylenebis (4-hydroxycoumarin) and hepatic vitamin A

It is generally believed that prothrombin is synthesized in the liver (Smith, Warner and Brinkhous, '37). The anticoagulant 3, 3′-methylenebis (4-hydroxycoumarin)[2] causes a hypoprothrombinemia when fed to animals, although it does not affect the clotting of blood in vitro (Campbell et al., '41). Vitamin K on the contrary tends to increase prothrombin activity, and in the rat, at least, can be used to overcome the effects of the anticoagulant (Overman et al., '42). Carvone, which stimulates the synthesis of vitamin C in the rat, also counteracts the effect of the anticoagulant (Baumann, Field, Overman and Link, '42). In the present experiments the anticoagulant, vitamin K, carvone and combinations of these compounds were added to the basal diet I low in vitamin A and their effect on hepatic vitamin A determined.

Twenty-six rats, 44 gm. in weight, were given 3 drops of halibut liver oil at the beginning of the experiment and the various low vitamin A diets (table 2) fed for 8 weeks. The anticoagulant was fed as a mixture

[2] We are indebted to Prof. K. P. Link for this preparation.

of 1 mg. incorporated into 1 gm. of diet I. This amount fed three times a week to the young rats resulted in pronounced hemorrhages in the subcutaneous areas of the face or from the nose, and four of nine animals died. Accordingly the dosage of anticoagulant was reduced to 1 mg. a week and gradually increased to the desired level as the surviving rats increased in weight.

As a source of vitamin K 50 mg. of 2 methyl 1, 4 napthoquinone were added per kilogram of diet I. The carvone was mixed with the low vitamin A ration at the expense of the dextrin in the amount of 2.5 gm. of carvone per kilogram of ration. No gross abnormalities were evident in any of the animals on the latter diets.

TABLE 2

The depletion of hepatic vitamin A in rats fed substances that affect prothrombin activity.

SUPPLEMENT TO A-LOW DIET I	NUMBER OF RATS	WEEKS ON EXPT.	WEIGHT OF RATS	DICUMAROL[1] PER RAT	VITAMIN A PER LIVER
				mg.	*μg.*
None	2	0	48	.....	728 (658–798)
None	4	8	207	.....	307 (270–364)
Dicumarol[1]	6	8	187	14–15	347 (264–484)
Vitamin K	2	8	163	.....	350 (340–360)
Dicumarol[1] and Vitamin K	2	8	164	9–10	347 (336–357)
Carvone	3	8	174	.....	313 (244–380)
Carvone and Dicumarol[1]	3	8	169	14	255 (243–267)

[1] 3, 3′ methylenebis (4-hydroxycoumarin).

Immediately after the ingestion of halibut liver oil, the animals averaged 728 μg. of vitamin A per liver. Control animals kept for 8 weeks on diet I, with no variations, retained 307 μg. of the vitamin in their livers at the end of this period. Animals receiving the 3, 3′-methylenebis (4-hydroxycoumarin) or vitamin K or a combination of these compounds retained 350 μg. of vitamin A per liver (table 2). Thus neither the anticoagulant nor vitamin K hastened the depletion of the hepatic vitamin A; in fact if anything, they appeared to retard it somewhat. Since both the anticoagulant and vitamin K affected vitamin A storage in the same way, it was evident that there was no parallelism between vitamin A storage and prothrombin activity. Carvone also appeared to be without effect upon vitamin A storage (table 2).

Effect of fat and choline

In the first series eighteen female rats 145 gm. in weight (± 20) were placed on three different rations low in vitamin A. Twelve of the

rats were fed a high fat diet IV consisting of extracted casein 5%, cystine 0.2%, yeast 5%, salts 4%, cottonseed oil 2%, hydrogenated cottonseed oil 30%, and dextrin 53.8%. Three other rats received the above ration supplemented with 0.3% choline. The remaining three rats were fed diet V which was low in fat as follows: casein 3.5%, cystine 0.15%, yeast 3.5%, salts 2.9%, cottonseed oil 1.5% and dextrin 88.5%. After 1 week each rat was given 2 drops of halibut liver oil, and 2 weeks later two of the animals on diet V were killed for analysis. Five of the other rats were continued on diet IV while the remaining five were fed the low-fat diet supplemented with 0.3% choline (diet VI). At the end of the fourth week all animals in the series were killed for analysis. For the quantitative determination of vitamin A the livers were allowed to stand overnight in alcoholic KOH at room temperature

TABLE 3

The effect of fat and of choline on the hepatic storage of vitamin A.

DIET	NUMBER OF RATS	WEEKS ON EXPERIMENT	WEIGHT BEGIN.–END	PER CENT LIVER FAT	VITAMIN A PER LIVER
			gm.	*fresh wt.*	*µg.*
High fat diet IV	2	3	147–164	31	337 (312–362)
High fat diet IV	5	4	150–156	40	381 (289–478)
High fat IV + 0.3% choline	3	4	148–159	3.4	395 (278–478)
Low fat diet V	3	4	149–157	24	311 (291–342)
High fat IV, 3 weeks, low fat + choline, diet VI 4th week	5	4	147–163	27	370 (286–518)

followed by 30 minutes on the steam bath. This procedure resulted in a more thorough saponification of the fatty livers in the series. Previous analyses had indicated that the values for vitamin A in very fatty livers are increased by 15% when a hot alcoholic saponification is employed in place of the usual cold aqueous solution of the tissue (Clayton, '42).

Except in the group fed choline throughout the experiment, the animals had typically enlarged, pale, and friable fatty livers. Rats fed the high fat diet IV for 4 weeks had a liver fat content of 40% based on the fresh weight, in contrast to a percentage of only 3.4% in the group fed an additional 0.3% of choline. Nevertheless both groups contained essentially the same amount of hepatic vitamin A (table 3) and in the entire series the variations in hepatic vitamin A within the various groups were usually greater than between groups.

The relationship between liver fat and hepatic vitamin A was also studied in ninety adult mice that were fed either a diet comparatively low in fat (diet I) throughout the experiment or a pair of diets alternating at weekly intervals. One of the latter diets was designed to effect a rapid accumulation of fat in the liver, while the other caused a rapid rate of depletion of the surplus liver fat. Uniform storage of hepatic vitamin A was assured by feeding 1 drop of halibut liver oil to each mouse at the beginning of the experiment. Thereafter all rations fed were free from vitamin A. Diet VII was high in fat and low in lipotropic factors, yet contained as much total protein as the

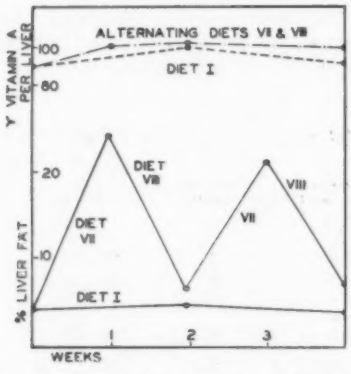

Fig. 1 Constancy of vitamin A in mouse livers of varying fat content.

The solid lines represent the percentages of liver fat; the broken lines, the micrograms of vitamin A per liver. Each circle represents the averages of at least five determinations made separately.

Diet VII was high in fat and low in lipotropic factors, viz. casein 5%, arachin 13%, cystine 0.2%, fat 32%, etc. Diet VIII was low in fat and high in lipotropic substances: casein 18%, choline 1%, etc. Diet I contained 18% of casein and 5% of fat.

other low A diets fed. It consisted of casein 5%, arachin 13%, cystine 0.2%, salts 4%, yeast 5%, hydrogenated vegetable oil 30%, cottonseed oil 2%, and dextrin 40.8%. Diet VIII, fed for the rapid removal of fat from the liver, consisted of diet I plus 1% of choline, the lipotropic effect of which was enhanced by the 18% of casein also present in the diet. Diets VII and VIII were fed alternately at intervals of 1 week.

The results are presented in figure 1. On the low fat diet I, the liver fat remained at approximately 4% of the fresh weight during the 4 weeks of the experiment. In contrast, the group fed diet VII (arachin) developed very fatty livers within 1 week (liver fat $=24\%$), and when diet VIII was fed to these animals most of the extra fat had disap-

peared from the liver 1 week later. Subsequent feeding of the arachin diet raised the percentage of liver fat to 21% and this extra fat was again removed from the livers after feeding diet VIII for 1 week longer. In spite of these very drastic changes in the percentage of liver fat, the vitamin A content of the liver remained essentially unchanged during the 4-week experiment. Furthermore, the amount of vitamin A in the livers of mice fed the alternating diets was essentially the same as in those fed diet I throughout the experimental period.

In agreement with Lease and Steenbock ('39) and contrary to the results of Thorbjarnson and Drummond ('38) our results all appeared to indicate that the retention of vitamin A in the liver did not depend upon the retention of fat by this organ. Since choline is very active in removing fat from the liver, it might be inferred that choline likewise has no effect on hepatic vitamin A. However, the experiments both with rats and mice were performed with adult animals, which are known to be relatively resistent to the effects of choline deficiency per se (Griffith, '40). Accordingly, the distribution of vitamin A was determined in young rats exhibiting very severe symptoms of choline deficiency.

Eighteen rats, 31–39 gm. in weight, were each fed 2 drops of halibut liver oil. They were then placed on a synthetic diet free from choline (Jacobi and Baumann, '42) and six of the animals received an additional 8.5 mg. of choline per day. On the second day three of the animals were killed for analysis of the vitamin A content of the liver, kidney, and other organs. After 5 days the animals on the low choline diet began to lose weight, and four of nine developed severe intraocular hemorrhages. On autopsy all of the deficient animals had enlarged kidneys, frequently bloody or mottled. In contrast the animals receiving the choline supplement remained in good physical condition, and no abnormalities of the kidneys were evident. In spite of these differences between groups the vitamin A contents of both livers and kidneys were essentially the same in the presence or absence of dietary choline (table 4). Contrary to the report of Popper and Chinn ('42) there was no evidence that the choline deficiency had altered the distribution between the liver and kidney of preformed stores of vitamin A. Evidence of a shift of vitamin A from the liver to the kidney may however have been suggested in another group of eight rats, 50 gm. in weight, fed the low choline diet (± choline) for 10 days plus 50 μg. of carotene per rat per day fed by dropper in cottonseed oil. The animals in both groups averaged 77 gm. in weight after 10 days without any visible symptoms of choline deficiency. Those on the deficient diet contained

only occasional traces of the vitamin per liver while 3.5 μg. of the vitamin were present per pair of kidneys. In contrast, the animals receiving choline averaged 3.9 μg. of vitamin A per liver and only 1.6 μg. per pair of kidneys. These amounts of vitamin A, however, were not much greater than the limit of error of the analytical method employed, and furthermore, the translocation of the vitamin was considerably less than that previously observed in the presence of dibenzanthracene (Baumann, Foster and Moore, '42).

TABLE 4

The distribution of vitamin A in the tissues of choline-deficient rats.

DIET	DAY	MICROGRAMS VITAMIN A			NUMBER OF RATS
		Liver	Kidneys	Organs[1]	
	2	260	3.5	2.7	3
Low choline	10	278	9.1	4.2	6
High choline	10	234	8.0	4.0	3
Low choline	20	234	5.3	1.8	3
High choline	20	233	6.0	2.3	3

[1] The organs included the lungs, spleen, adrenals and approximately 2 ml. of blood from each rat.

DISCUSSION

The hepatic storage of vitamin A was found to be unaltered by the most potent carcinogenic agent known for the liver of the rat; it was unaffected by factors that either depress or enhance the formation of prothrombin; and it remained essentially the same whether the diets fed were high or low in fat. Large amounts of fat could be removed from the liver without any accompanying loss of vitamin A from this organ. Nor did the retention of vitamin A by the liver appear to depend upon the presence of choline in the diet. These results all suggest that vitamin A is stored in the liver and released therefrom by a mechanism that is comparatively independent of other processes taking place in this organ. A similar conclusion is implicit in the results of Abels et al. ('42), who observed that dibenzanthracene, which seriously interferes with the retention of vitamin A by the liver, failed to affect the esterification of cholesterol, the formation or the conjugation of glucuronic acid, the formation of prothrombin or of other proteins of blood plasma, or the storage capacity of the liver for total fat, phospholipid or riboflavin. It would thus appear that many chemical reactions proceed in the liver independently of one another, and that a mild derangement of one does not necessarily interfere with the others.

In severe hepatic failure, on the contrary, it would obviously be possible for more than one process to be affected.

SUMMARY

The hepatic storage of vitamin A appeared to be relatively independent of other biochemical processes taking place in the liver. p-Dimethylaminoazobenzene, a potent agent for the production of liver tumors in the rat, failed to alter the rate of depletion of preformed stores of the vitamin, even on diets optimal for the carcinogenic action of the dye.

3, 3-Methylenebis (4-hydroxycoumarin), which depresses prothrombin synthesis, and vitamin K, which promotes it, were equally without effect on the rate of depletion of hepatic vitamin A. Carvone, likewise, was without effect.

The retention of vitamin A by the liver of the rat was essentially the same on diets either very high or low in fat; the rate of depletion of the vitamin from very fatty livers was the same as that from normal livers. In the mouse very high percentages of liver fat were repeatedly accumulated and "flushed out" without effect on the retention of hepatic vitamin A. The normal distribution of vitamin A was observed in tissues of young rats with severe symptoms of choline deficiency.

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PHYSIOLOGICAL AND BIOCHEMICAL FUNCTIONS IN NORMAL YOUNG MEN ON A DIET RESTRICTED IN RIBOFLAVIN [1]

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(Received for publication August 16, 1943)

In this laboratory we have been concerned with the possible limitation of human work performance by restriction in dietary elements such as may be encountered in war time, particularly in military operations. The vitamins of the B complex have been prominently mentioned in this connection in certain circles. Of these vitamins thiamine, riboflavin and niacin occupy first place by nature of their undoubted importance in fundamental enzyme reactions involved in carbohydrate and presumably muscle metabolism.

We have previously reported our inability to detect any advantage for work performance of "supercharging" with these vitamins over periods of 6 to 10 weeks (Keys and Henschel, '42). Studies on young men maintained for 10 to 12 weeks on considerably less than half the National Research Council recommended allowance for thiamine failed to disclose any limitation on work performance and related metabolic functions (Keys, et al., '43). The present article is a report on similar studies with an intake of riboflavin restricted to about one-third the N.R.C. recommendation for a period of 22 weeks with additional control periods totaling 15 weeks.

SUBJECTS AND REGIME

The subjects were six men students free from signs and histories of metabolic, nutritional and digestive peculiarities. They were especially selected for reliability and cooperation. During the entire experimental

[1] This work was supported in part under the terms of a contract, recommended by the committee on Medical Research, between the Regents of the University of Minnesota and the Office of Scientific Research and Development. Important financial assistance was also provided by the Nutrition Foundation, Inc., by the U. S. Cane Sugar Refiners' Association, N. Y., by the Corn Industries Research Foundation, N. Y., by the National Confectioners' Association, Chicago, and by Swift and Co., Chicago. Merck and Co., Inc., provided generous supplies of pure vitamins. Valuable aid was given by the Work Projects Administration under the University of Minnesota Project no. 8760, Subproject 380.

period with the exception of a few pre-arranged days and a part of one control period the subjects received all meals in the laboratory and resided in rooms nearby. They were not under immediate supervision except at meal times and 1 or 2 days each week but they were thoroughly aware of the importance of conforming to the limitations of the program and were undoubtedly anxious to fulfill their roles as cooperators. Their adherence to the conditions was demonstrated by the results of urine analyses which were made weekly and by the reports of their classmates.

Once each week on the average the 24-hour urine was collected and from time to time this was succeeded by a 24-hour "saturation test" collection. Once each week the subjects carried out a series of work tests which involved, successively, 30 minutes' bed rest, 60 minutes' walk on the treadmill at 3.85 miles per hour and 10% grade, 2 minutes' standing rest, 10 minutes' bed rest, 60 seconds' run on the treadmill at 9.5 miles per hour and 10% grade, bed rest for 25 minutes. Every other week venous blood samples were taken at the end of the first bed rest and at 8, 12 and 25 minutes after the end of the work in the final bed rest. Pulse rates were taken during the first bed rest, during the final 10 minutes of the walk, for the first 2 minutes (standing) after the walk, and at 1 and 2 minutes after the end of the run. On alternate weeks the battery of psychomoter tests was run through once during the first 10 minutes of walk on the treadmill and again in the last 10 minutes of the walk. All test procedures were made in a controlled environment suite, temperature 78°F., humidity 50% relative saturation. Standard clothing in all tests consisted of cotton gymnasium shirts and shorts, socks and shoes.

Clinical examinations were made each month; these included slit lamp observations of the corneas and very careful examination of the skin, lips, mouth and tongue. Electrocardiograms and measurements of basal metabolism were made before and after as well as at intervals during the restricted regime.

All subjects were trained and standardized with the several tests and procedures for 3 weeks preceding the restricted diet period. During this time the men subsisted on the ordinary diet furnished by their boarding houses. Three of the men (group A) were maintained on a restricted diet for 84 days, and the other three men (group B) were continued on for a total of 152 days on the low B_2 intake followed successively by 26 days on a high (11 mg.) intake, 6 days on the low regime, 36 days on a relatively normal B_2 intake and finally 8 days on the low intake.

DIET

All the meals were prepared in the laboratory diet kitchen by a dietitian and assistants. Strict menus were followed and all portions were weighed. An extra identical meal was prepared and used for analysis. These were rapidly and carefully ground and stored at —25°C. for daily analysis.

The basic composition of the diet provided a "normal" balance — 55 to 75 gm. (av. 60 gm.) of protein and 37% of the calories as fat. Milk and eggs were not used and only very small amounts of meats and green vegetables were allowed. Unfortified flour was used for all breads and pastries. Daily supplements, in capsules, provided 1.0 mg. thiamine, 10 mg. niacinamide, 50 mg. ascorbic acid. The total daily caloric intake was first set at 3000 Cal. This proved slightly less than required to maintain body weight so the amount was increased, by additional breads, carbohydrates and a small amount of fat, to an average of 3160 Cal.; these additions sufficed to maintain body weights in all but one subject, C. M., who lost 12 pounds (177 to 165) in the 5-month period on the low B_2 regime. The final average caloric intake then was about 3150 $\pm$ 100 Cal.

The riboflavin content of the diet was planned to be about 1.0 mg. per day or slightly less. Actually, the analyses for the first 3 weeks indicated a riboflavin content about 20% greater than calculated from the available "book" values. Accordingly the diet was then more severely restricted so that the average daily intake for the entire 152 days was 0.99 mg. or a grand average of 0.31 mg. of B_2 per 1000 Cal. The riboflavin intakes for different periods are indicated in table 1. Foods for representative days are given in table 6.

METHODS

Great pains were taken to ensure the validity of all riboflavin analyses. Riboflavin in the urine was estimated both by the microbiological method of Snell and Strong ('39) and by a modification of the fluorometric method of Connor and Straub ('41). The latter procedure proved to be considerably more satisfactory and was relied upon exclusively for the last 3 months. For the foods the microbiological method of Snell and Strong was modified according to the nature of the material, the criteria being the production of a crystal clear filtrate, the recovery of known additions, and good agreement between duplicates (Andrews, et al., '42). Ether extraction, diastatic treatment and filtration at pH 4.5 were variously applied to achieve these ends.

The 24-hour urine was collected in amber glass bottles containing 5 ml. of glacial acetic acid and 3 ml. of toluol. The samples were stored at 3°C. while awaiting analysis.

Venous blood, drawn from a vein in the antecubital fossa with a minimum of stasis, was analyzed for lactate by the method of Friedemann, Cotonio and Shaffer as modified by Edwards ('38). Pyruvate was estimated by the method of Lu ('39) as modified by Friedemann and Haugen ('43). Blood sugar was measured by the method of Folin and Wu ('20) using cadmium hydroxide as the precipitating agent. Hemoglobin was estimated as oxyhemoglobin with a photoelectric colorimeter.

Measurements of flicker fusion frequency and of grip and back strength were made before and after the 60-minute walk periods. Manual speed was tested with Brozek's ('43) ball and pipe method, motor control by tracing a stylus through a "maze," and speed of small movements by the tapping test as used previously (Keys, et al., '43). These last three tests were carried out during actual work on the treadmill.

RESULTS

The data are too extensive for detailed presentation here. Fortunately there was relatively little individual variation between the several subjects so treatment by averages is justifiable as well as expedient. The two groups were closely similar in all respects up to the time of discontinuance of group A so only the measurements with group B will be given in some of the tables. It must be emphasized, however, that the data cited on group A could apply almost identically to group B up to the end of their participation (84 days on the restricted diet).

Riboflavin excretion

Riboflavin excretion in the 24-hour urine for the restricted period is summarized in table 1.[2] During the first few weeks on the restricted regime the riboflavin excretion decreased progressively but after about a month and a half the excretion appeared to be stabilized at from 10 to 14% of the intake. When extra test doses were introduced their per cent of recovery was very similar. The indication of slightly smaller recovery of test doses in the table cannot be given any weight because of the technical difficulty of the procedure.

[2] Results from one subject (E.L.F.) are omitted because we were consistently baffled in the attempt to get reliable results on his urine; in particular the microbiological and chemical methods were frequently in disagreement on his urine.

When, after 5 months on the restricted regime, the daily intake of riboflavin was raised to 11.2 mg., the urinary excretion increased abruptly so that after 1 week an average of 54% (45, 58 and 62% in the three men tested) of the intake appeared in the urine. Return to the low riboflavin intake after 26 days of this supercharging brought the excretion down to an average of 38% of the intake on the fifth day. A saturation test on the sixth day showed 19.7% recovery. Finally, return to fraternity house diet for 5 weeks resulted in excretions of 30 to 100 micrograms of riboflavin per day after an additional week on a measured daily intake of 0.95 mg.

TABLE 1

Average riboflavin intake and 24-hour excretion. Intake values are averages, in miligrams per day, for the days indicated. Excretion values, in micrograms in 24 hours, are the averages of two to four measurements for each man in the indicated periods. "Test recov." shows the elevation of the 24-hour excretion resulting from administration of 1.0 mg. of crystalline riboflavin.

Days	4–8	15–22	28–38	48–55	63–72	78–85	97–104	110–119	126–134	142–149
Intake	1.32	0.97	1.04	0.98	1.02	0.90	0.87	0.89	0.82	0.90
Excretion:										
Subj. C M	...	155	155	147	120	139	162	87	98	108
Subj. L E	422	263	251	189	145	182	119	108	112	123
Subj. C T	359	175	149	101	81	96	77	62	98	89
Subj. F T	230	294	179	188	159	153	...	...	...	...
Subj. G W	213	186	135	81	83	82	...	...	...	...
Average	308	215	174	141	118	130	119	89	103	107
As % intake	23%	22%	17%	14%	12%	14%	14%	10%	12%	12%
Av. test recov.	...	...	...	...	...	9.1%	13.4%	...	...	8.0%

The data clearly show that the body can accumulate a reserve of riboflavin but that this reserve was not great and was lost in a few weeks. It is important that, in the present series, an equilibrium between intake and output was established on the low regime and there were no signs of progressive alteration in this equilibrium after the first 2 or 3 months.

Eye examinations

Five out of the six subjects initially showed minor peculiarities in the slit lamp examinations. These included whitish, granular, sub-epithelial deposits at the limbus and the center of the cornea and slight peripheral vascularization at the limbus (4 to 5 arcades, 3 to 8 mm.). There was no change in any of the eyes at any time during the period of the restricted riboflavin intake nor afterwards in the restoration control periods. In all other respects also the eyes were apparently unaffected by the diets.

Other clinical observations

The skin, mouth and tongue of all subjects remained apparently entirely normal at all times. No signs of cheilosis, stomatitis or scaling at the nasolabial folds appeared. All other observations were constantly negative. At no time was there any evidence of alteration in gastro-intestinal, cardiovascular or neuromuscular functions. The electro-cardiograms were unchanged in all men in all leads throughout the entire period of observation. The direction, potential and duration of the several components of the E.C.G. were examined.

Work performance

In any work performance measurements, it is essential that the work tasks and conditions be rigidly standardized and the training factor must be held constant. In the present study it was not possible to maintain absolute constancy of general muscular training and rest. These studies were started at the end of the summer when these subjects, like college students in general, were in a better state of physical training than at other times of the year. About a month after the start of the low B_2 regime the subjects began to "cram" for their comprehensive examinations with the result that recreational muscular exercise and hours of sleep were cut to a minimum for about a month. The effects of these conditions were reflected in some of the measurements in the performance studies.

The heart rate in standard rest was remarkably constant throughout. The heart rate in work, because of its central position in the homoeostatic-mechanisms of the body, is a sensitive and valuable criterion of work capacity. In the 60-minute walk difficulties with the cardiotachometer spoiled the pulse rate records in work for the initial control period and the first few weeks on the low B_2 intake. Thereafter, however, the rates were substantially constant for about 2 months with a grand average of 141 and weekly maximum and minimum of 144 and 137 beats per minute. Several weeks after the end of the comprehensive college examinations there was a slight improvement in all men and this new level of work pulse rate (average 131) was maintained with substantially no change for the balance of all the studies, including those in the "restoration control" period.

The average heart rates for group B in recovery following anaerobic work are given in table 2. It is clear that after about a month on the low B_2 regime the men exhibited a moderate decline in capacity for this type of work which persisted until the end of the college examination period

and subsequently there was small improvement in the 1-minute pulse and a more marked return toward the original values in the 2-minute pulse. There were no changes in the "restoration control" period.

Average values for blood glucose, lactate and pyruvate in rest and after work in group B are given in table 3. The glucose and pyruvate values were remarkably constant in all periods but there are some

TABLE 2

Average pulse rates, group B, after 30 minutes' bed rest and at 1 and 2 minutes after standard anaerobic work. "Before" represents the control period before B_2 restriction and after" is the restoration control after 17 days on a daily intake of 11 mg. of B_2. Other column headings refer to the number of days on the restricted regime.

CONDITION	BEFORE	22	36	50	64	78	105	119	134	148	AFTER
Rest	66	67	68	65	69	68	69	71	69	66	72
1 Min.	142	145	159	159	166	153	153	155	151	152	160
2 Min.	132	133	148	152	145	135	131	137	133	136	136

TABLE 3

Average values, group B, for blood analyses in rest and at 12 and at 25 minutes after standard anaerobic work. All values in ml. per 100 ml. of whole blood. "Before" represents the control period before B_2 restriction. "After" represents the "restoration" control after 17 days on a daily intake of 11 mg. riboflavin. The days noted refer to number of days on the restricted regime.

PERIOD	REST			12 MIN. RECOVERY			25 MIN. RECOVERY		
	Glucose	Lactate	Pyruvate	Glucose	Lactate	Pyruvate	Glucose	Lactate	Pyruvate
Before	79	9	0.9	74	47	3.5	75	43	2.5
22 days	81	8	0.8	75	42	2.5	72	31	2.3
36 days	73	14	0.7	78	48	2.5	76	33	1.9
50 days	76	14	0.7	74	55	2.7	68	41	1.9
64 days	82	13	1.0	76	55	2.7	72	44	1.9
78 days	79	12	0.9	80	62	3.2	81	39	2.4
105 days	79	10	0.8	76	64	3.0	73	35	2.4
119 days	76	7	0.8	75	57	3.1	71	34	2.2
134 days	78	7	0.7	78	61	3.4	75	38	2.5
148 days	79	7	0.9	78	56	3.6	71	38	2.5
After	79	7	1.0	81	55	3.5	73	28	2.6

points to be noted in the lactate concentrations. There was a slight elevation in the resting lactate from the thirty-sixth to the seventy-eighth day; this period covers the "cramming" and examination period and several weeks afterwards. We have noted the temporary disturbance in the pulse rates in this period. A slightly greater lactate accumulation at 12 minutes after work appeared after about 6 weeks on the

restricted regime. This change was small and not progressive. Its explanation is questionable but presumably it reflects a loss of the benefits of the summer physical activity.

The results of the simple muscle strength tests are notable for their really surprising constancy. For example, the grip strength of one subject (C. T.) did not vary from week to week beyond the range of 41 to 44 pounds and that of another subject (C. M.) beyond the range of 66 to 72 pounds. The weekly averages of grip strength for group B varied from 51 to 54 pounds in four preliminary control periods, from 52 to 56 pounds in the entire restricted diet period and from 53 to 54 in the "restoration control" period. This is interesting in view of the fact that there is evidence that grip strength responds to slight changes in condition, incipient colds etc., (Metheny, '40). The back dynamometer results were only slightly more variable and equally failed to disclose any relation to the riboflavin intake.

TABLE 4

Average results of psychomotor tests, group B. "Tapping" is speed of tapping in contacts made in the first 10 seconds ("A") and in the third 10 seconds ("B"). "Pipe" is the ball and the pipe test, results in number of balls passed through the pipe in 60 seconds. "Maze" refers to the motor control tests; "A" gives the number of contacts per circuit and "B" is the total duration of those contacts in units of 1/120 seconds. F. F. F. is the flicker fusion frequency in flickers discriminated per second. Note that low scores are "good" in the maze test (A and B) and "poor" in all other tests. "After" refers to measurements made after 26 days of supplementation (11.2 mg. B_2 per day).

TEST	BEFORE	WEEKS ON LOW B_2 REGIME										AFTER
		2	4	6	8	10	14	16	18	20	22	
Tapping A	68	70	69	70	69	72	72	72	72	74	74	74
Tapping B	58	60	60	60	61	59	63	62	62	63	64	63
Pipe	64	64	64	63	63	64	67	68	66	66	68	68
Maze A	86	83	74	72	77	77	71	61	66	56	55	56
Maze B	478	539	445	461	449	454	439	452	378	338	344	335
F. F. F.	...	...	61	61	60	60	60	60	63	62	62	61

Psychomotor results

The average results of the psychomotor tests with group B are given in table 4. Entirely similar results were obtained with group A and with the different subjects considered individually. There were some progressive training effects with the motor control (maze) test and smaller training changes in the pipe and tapping tests. These training

effects were entirely normal as judged by extensive studies on persons on other diets. The flicker fusion frequency, which has no appreciable training element, was not measured in the preliminary control period but all the measurements made were extraordinarily constant in each individual throughout the entire subsequent period.

Other measurements

There was a tendency for the hemoglobin concentration in the blood to rise slightly during the period of riboflavin restriction. This regressed on the return to a normal diet and was seen in both groups A and B (table 5). The interpretation of this is obscure.

TABLE 5

Average values for glucose tolerance, for blood hemoglobin and basal metabolism. Glucose in milligrams and hemoglobin in grams per 100 ml. of blood. B.M.R. as % of Mayo Clinic Standards.

GROUP	PERIOD	GLUCOSE TOLERANCE				Hb	B.M.R.
		Basal	30 MIN.	60 MIN.	90 MIN.		
A	Before	69	107	111	81	15.3	.....
A	84 Days	72	117	118	91	16.0	.....
B	Before	76	124	99	92	15.7	—13%
B	84 Days	76	110	77	74	16.0	—15%
B	148 Days	75	100	83	86	15.8	—13%
B	After	78	121	87	89	15.5	—16%

The basal metabolism was remarkably constant at all times in group B (table 5); it was not measured in group A. All subjects at all times had B.M.R. values slightly below the "normal" averages of the Mayo Clinic but this is usual in this laboratory where the subjects are trained and more complete relaxation is customarily attained.

Average glucose tolerance results are given in table 5. The results differ somewhat in groups A and B but there was a distinct tendency for all the men in group B to exhibit a depressed tolerance curve during the period of riboflavin restriction and for this to return toward the original control level on the resumption of a normal diet. Again the significance, if any, of these changes cannot be surmised here. The basal blood sugar values were very constant throughout. The relatively low values reflect the method used which eliminates more non-glucose reducing substances than the more common clinical methods.

TABLE 6

Foods for representative days, together with riboflavin analyses and total calories. "W.K." is canned whole kernel corn; "lettuce, fr." is lettuce with french dressing. All cake, bread, pie, candy are made with special recipes using unenriched flour, little or no milk and minimal amounts of eggs. Cake and bread were fortified with purified casein and calcium phosphate and iron salts. "Cream, 25%" was prepared from 70% butter fat cream and salt solution.

DEC. 1, 1942	FEB. 2, 1943	FEB. 11, 1943
100 gm. Grapefruit juice	100 gm. Apple sauce	100 gm. Grapefruit
20 gm. Corn flakes	20 gm. Puffed rice	20 gm. Puffed rice
70 gm. Butter	80 gm. Butter	80 gm. Butter
100 gm. Cream, 25%	100 gm. Cream, 25%	100 gm. Cream, 25%
180 gm. Bread	240 gm. Bread	20 gm. Gran. sugar
20 gm. Gran. sugar	20 gm. Gran. sugar	180 gm. Bread
100 gm. Grape jelly	150 gm. Cranberry jelly	100 gm. Apple jelly
40 gm. Fudge	40 gm. Fudge	200 gm. Spaghetti
200 gm. Spaghetti with lettuce	30 gm. Hamburger	30 gm. Meat ball
25 gm. Bacon	50 gm. Fried onions	100 gm. W.K. corn
50 gm. Lettuce, fr.	150 gm. Potato salad	50 gm. Lettuce
100 gm. W.K. corn	50 gm. Lettuce	100 gm. Jello
100 gm. Gingerbread	100 gm. Peach preserve	50 gm. Cookies
100 gm. Hamburger	80 gm. Swiss steak	80 gm. Veal roast
150 gm. Baked potato	150 gm. Mashed potatoes	150 gm. Mashed potatoes
100 gm. Boiled onions	100 gm. Boiled cabbage	100 gm. Boiled carrots
50 gm. Raw carrots	100 gm. Chocolate cake	50 gm. Cranberry sauce
100 gm. Strawberry preserve		100 gm. Angel food cake
Final wet wt. = 1605 gm.	Final wet wt. = 1525 gm.	Total wet wt. = 1230 gm.
Total calories = 3090	Total calories = 3250	Total calories = 2810
Total B_2 = 0.91 mg.	Total B_2 = 0.92 mg.	Total B_2 = 0.66 mg.

DISCUSSION

There have been relatively few prolonged studies on human beings maintained on known restricted intakes of riboflavin. Physical performance and other objective functional tests have not been reported before. In other respects it is useful to compare the present findings with those of Sebrell, et al. ('41) and the report of Williams, et al. ('43) which appeared just as the present research was being completed. In both of these studies the subjects were women inmates of institutions who were maintained at considerably lower caloric levels than our subjects. The Mayo Clinic subjects were particularly sedentary. If the riboflavin intake is considered in relation to total calories our subjects received about 50% more than Sebrell's subjects and about 12% less than those of Williams, et al. If total riboflavin per day is considered, then our subjects received about twice as much as Sebrell's subjects and almost 50% more than Williams' subjects. Further, our subjects went on the restricted regime from a college boarding house diet while

Sebrell's subjects may have been chronically somewhat malnourished and Williams' subjects were fortified by a prolonged high B_2 intake.

In any case the urinary excretions of riboflavin were of the same order of magnitude in all three studies. The average proportion of the dietary riboflavin which appeared in the urine was about 15% in Sebrell's subjects, 14% in those of Williams, and 12% in our subjects. The saturation test methods were not strictly comparable but it appears that our subjects showed somewhat lower apparent "saturations" than the Mayo Clinic subjects. The present results indicate that when the test dosage is of the same order of magnitude as the dietary intake and when the latter is low and fairly constant, then the proportion of total riboflavin intake which appears in the urine is relatively independent of the test dose. We agree with Najjar and Holt ('41) that the erratic results in the saturation tests reported by Axelrod, et al. ('41) may be explained by excessive test dosage.

Aside from urinary excretions no evidence whatever of riboflavin deficiency appeared in either our subjects or those of Williams, et al. Six out of the ten subjects in the series of Sebrell, et al., developed cheilosis in 89 to 232 days. The specificity of this lesion has been seriously challenged (e.g. Machella, '42; Machella and McDonald, '43) and we should note that the diet used by Sebrell, et al., may have been deficient in other B vitamins; pyridoxine has been reported to heal cheilosis in many cases (Smith and Martin, '40; Machella, op. cit.).

Estimation of the riboflavin requirement of man and the diagnosis of riboflavin deficiency are in a peculiarly unsatisfactory state. Youmans ('41) noted that the diagnosis of ariboflavinosis should be confirmed by objective changes induced by therapy with the vitamin. The situation has improved little in the succeeding 2 years. The extreme view that all capillary infiltration of the cornea indicates active or chronic riboflavin deficiency (Wiehl and Kruse, '41; Wiehl, '42; Borsook, et al., '43) appears to go far beyond the known facts. If we accept this theory then we should conclude that five out of six of our subjects suffered from slight riboflavin deficiency when they started the experiment. In any case neither the deficiency regime nor the "restoration" control period produced the slightest change in the eyes.

In the absence of other criteria urinary excretion and "saturation" tests have been used. Williams, et al. (op. cit.) state that their data show "unmistakable depletion of tissue stores of riboflavin" (p. 376), from which they conclude that the intake of 0.35 mg. per 1000 Cal. does not meet "requirements." Their data actually suggest that the tissue

stores of riboflavin in the first month or so of the restricted diet were larger than during the subsequent period and therefore indicate that a prolonged period of high riboflavin intake induces some temporary accumulation of the vitamin. Our results are in agreement. This does not necessarily mean that the body is then "depleted" in the sense of "starved" or "exhausted," when such an accumulation is reduced, nor does it necessarily follow that functional requirements of the body include such an excess store of riboflavin in the tissues. It is not proposed to discuss here the proper meaning of those popular terms "requirements" and "sub-clinical deficiency." The principal purpose of the riboflavin excretion measurements in the present study was to guarantee the estimate of the state of riboflavin nutrition. The results show that the riboflavin nutrition was as indicated by the diet and the food analyses. Both dietary and excretion data agree that the true intake of riboflavin was far lower than various "recommendations" and therefore did not meet requirements in this sense. But the main point of this work was to measure, by the most sensitive and objective means we could devise, the functional capacity of the body under these conditions. The results indicated that, within the limitations of time and subjects used, the sole disadvantage of the low intake which we found was in the small amount of riboflavin excreted.

The present results indicate that normal young men suffer no physiological or clinical handicap by restriction to an intake of riboflavin of 0.31 mg. per 1000 Cal. for a period of 5 months. Longer periods and very young or aged people may be another matter. However we are unaware of evidence on these points. Undoubtedly such a restricted diet does not provide a body reserve as large as would result from a greater intake.

Comparisons with animal experiments are of doubtful utility but we may note that dogs may fail to show symptoms for a long time on diets extremely low in riboflavin. Of the three dogs studied for about a year by Fraser, Topping and Isbell ('40), one developed symptoms in 7½ months, another some months later and the third never showed any abnormalities within the period of 11½ months of observation. The diet provided these dogs contained about 0.1 mg. of riboflavin per 1000 Cal.

SUMMARY

Studies were made under controlled conditions on six normal men students subsisting on a diet restricted in riboflavin but otherwise adequate. The restricted regime was preceded by 3 weeks of control

observations. Three of the men remained on the restricted intake for 84 days; the other three men were on the low regime for 152 days. The latter group was also studied during 12 weeks of "restoration" after the low regime. The diet actually eaten was directly analyzed for riboflavin. It averaged 0.99 mg. per day (3150 ± 100 Cal.) or 0.31 mg. per 1000 Cal.

Standardized tests covered 60 minutes of hard work on the treadmill, an anaerobic work test, glucose tolerance, tests of muscle power and several psycho-motor tests. Observations included pulse rates in rest, work and recovery, metabolism, blood lactate, glucose, pyruvate and hemoglobin, and urinary excretion of riboflavin with and without "saturation" test doses. Clinical examinations included slit lamp and other special observations. Observations and tests were made every other week except for the saturation tests, glucose tolerance and eye examinations, all of which were made about once a month. Analyses of urinary excretion were made each week.

The 24-hour urinary excretion of riboflavin averaged 12% of the dietary intake on the low regime and the recovery of saturation test doses of 1.0 mg. was very similar. After the first few weeks there were no progressive changes in either "regular" or "saturation" excretion.

All other results were essentially negative and constant. No clinical changes occurred. Work performance and the bodily responses to work were essentially unaffected by the dietary alterations.

It is concluded that normal young men suffer no physiological handicap from subsistence for at least 5 months on a diet providing 0.31 mg. of riboflavin per 1000 Cal. (0.99 mg. per day in balance at 3150 Cal.).

ACKNOWLEDGMENTS

We are grateful to Dr. John S. Andrews and his associates for the care given to the large number of riboflavin assays made in the Research Laboratory of General Mills, Inc. General and special medical examinations were made by Dr. C. A. McKinley in the Student Health Service. This work depended in large measure on the constant assistance of several members of the Staff of this Laboratory: Miss Angie Mae Sturgeon, Head Technologist, Miss Doris Doeden and Mr. Howard Condiff, Assistant Chemists, Miss Catherine Carroll, Technician and particularly Miss Evelyn Pearson, Dietitian and Mrs. Nedra Foster, Assistant Detitian.

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THE INFLUENCE OF THE PROTEIN CONTENT OF THE DIET UPON FAT DIGESTIBILITY[1]

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(Received for publication August 19, 1943)

In the course of certain nutrition studies at this laboratory in which two levels of protein were being fed to rats, it was noticed that the groups receiving the lower protein diets excreted feces that were consistently light in color. Since no information was available which offered an explanation for this difference, measurements of fecal fat were considered advisable. Preliminary determinations showed that the fat content was considerably elevated in the feces of the rats receiving the lower protein diets. Therefore, a more critical study of fat digestibility was undertaken.

EXPERIMENTAL

Two groups of adult rats (approximately 200 days of age) that had been raised from the time of weaning on semi-purified diets were kept in large-mesh wire bottom cages that were elevated several inches above a metal tray covered with heavy paper. The food was placed in small glazed clay feeders and the rats were allowed to eat ad libitum. Food intake was measured over 3- and 4-day periods. Spillage was noted, but was in no case serious enough to warrant corrections. When changes in the type of fat were made the new diets were fed for 1 week prior to starting collections. Feces were collected for the same periods and dried in an oven at 110°C. The fat was extracted with low boiling petroleum ether after saponification, acidification with hydrochloric acid and dilution with water. In calculating the fat digestibility no correction was made for endogenous fat excretion.

The diets contained either 12 or 28% casein, 5% yeast, 4% salt mixture [a modification of the mixture of Hubbell, Mendel and Wakeman ('37) as described by Clausen, Barnes and Burr ('43)], 18% of the test fat, and sucrose to make up the balance. Three drops of a mixture

[1] Aided by grants from the Medical Research Fund of the Graduate School and the National Dairy Council.

of 2 parts cod liver oil and 1 part wheat germ oil were administered by mouth with an eye dropper twice weekly. All diets were kept in 1-gallon glass jars and stored in a refrigerator. The fats studied were a steam rendered lard (melting point 33–35°C.); a specially stabilized lard containing 8 to 12% lard which had been hydrogenated to an iodine value of approximately 30 (melting point 39–41°C.); a commercially packaged butter which was purchased during the winter months (melting point of the butterfat 26–28°C.); and an experimentally developed butter from cows which had been fed a diet known to cause an increase in the hardness of the butterfat and which in addition contained 5% completely hydrogenated vegetable fat. The melting point of the fat from this special butter spread was 39–41°C. Additional constants of the test fats are shown in table 1. Melting points were measured with

TABLE 1

Constants for the different fats.

TYPE OF FAT	IODINE VALUE (WIJS)	THIOCYANOGEN VALUE	SAPONIFICATION VALUE	REICHERT MEISSL NUMBER
Steam lard	65.50	56.02	194.0	....
Special lard	60.64	50.93	194.0	....
Standard butter	32.08	35.92	224.0	33.42
Special butter spread	39.40	33.50	213.0	20.93

a special melting point determination "hot-plate". Iodine, thiocyanogen, saponification and Reichert Meissl values were determined by the methods of the American Oil Chemists' Society ('41). Four groups of male and four groups of female rats were used in this study. They had been raised from the time of weaning on either the lower or higher protein diets containing either the specially stabilized lard or butterfat from the commercially packaged butter. Growth was normal as judged by comparison with stock animals, but the rats receiving the higher protein diet grew at a slightly more rapid rate. After making digestibility studies on these groups the rats receiving the special lard were fed a diet containing steam rendered lard. The rats which had been receiving butterfat were given diets containing the special butter-spread. One week after making these changes fecal collections were started.

RESULTS

The digestibility data are shown in table 2. In every case the rats receiving the lower protein diet had an increased amount of fecal fat. Perhaps this does not offer a complete explanation for the very light color of the feces from these rats, but it does indicate that the fat content

TABLE 2

Fat digestibility and protein content of the diet.

GROUP NO.	TYPE OF FAT	COLLECTION NO.	NO. OF RATS	FAT INTAKE [1]	WEIGHT OF FECES [1]	FECAL FAT	FAT ABSORBED [1]	DIGESTIBILITY [2]	NO. OF RATS	FAT INTAKE [1]	WEIGHT OF FECES [1]	FECAL FAT	FAT ABSORBED [1]	DIGESTIBILITY [2]
				FEMALES						MALES				
				gm.	gm.	%	gm.	%		gm.	gm.	%	gm.	%
1. Low protein	Steam lard	1	7	2.12	0.68	5.14	2.08	98.0	6	2.34	0.81	7.47	2.28	97.5
		2	7	1.88	0.54	4.91	1.85	98.4	6	2.05	0.86	7.25	2.00	97.5
		3	7	1.30	0.46	5.81	1.27	97.6	6	1.89	0.59	4.85	1.85	97.8
Average				*1.77*	*0.56*	*5.28*	*1.73*	*98.0*		*2.09*	*0.75*	*6.52*	*2.04*	*97.6*
2. High protein	Steam lard	1	5	2.34	0.94	0.35	2.33	99.9	6	2.50	0.88	2.57	2.47	98.8
		2	5	1.51	0.59	0.29	1.50	99.9	6	2.27	0.89	2.40	2.25	99.1
		3	5	1.80	0.69	2.06	1.79	99.5	6	2.23	0.92	5.79	2.18	97.8
		4	5	1.80	0.68	3.33	1.78	98.9	6	2.57	0.92	5.50	2.52	98.0
Average				*1.86*	*0.73*	*1.51*	*1.85*	*99.5*		*2.39*	*0.90*	*4.06*	*2.35*	*98.4*
3. Low protein	Special lard	1	7	1.71	0.52	22.90	1.59	93.1	6	1.91	0.52	24.46	1.78	93.4
		2	7	1.49	0.60	15.85	1.40	94.0	6	1.46	0.75	12.59	1.35	92.6
		3	7	2.36	0.57	19.27	2.25	95.4	6	0.95	0.82	14.51	0.83	87.4
		4	7	1.71	0.61	18.43	1.60	93.6	6	2.05	0.78	15.39	1.93	94.2
Average				*1.82*	*0.57*	*19.11*	*1.71*	*94.0*		*1.59*	*0.72*	*16.74*	*1.47*	*91.9*
4. High protein	Special lard	1	6	1.57	0.61	12.50	1.49	94.7	7	2.14	0.87	15.10	2.01	94.0
		2	6	1.37	0.60	7.91	1.32	96.4	7	1.89	0.91	9.32	1.81	95.7
		3	6	1.03	0.66	3.52	1.01	98.1	7	2.23	1.02	10.40	2.12	95.1
		4	6	1.49	0.71	7.61	1.44	96.7	7	2.04	0.99	6.00	1.98	97.0
Average				*1.36*	*0.64*	*7.88*	*1.31*	*96.7*		*2.07*	*0.95*	*10.20*	*1.98*	*95.4*
5. Low protein	Standard butter	1	6	1.04	0.53	22.14	0.92	88.5	4	0.97	0.62	28.98	0.79	81.5
		2	6	2.11	0.63	23.51	1.96	93.0	4	1.95	0.75	18.98	1.81	92.9
		3	6	1.74	0.83	10.92	1.65	94.8	4	2.47	1.27	12.81	2.31	93.6
Average				*1.63*	*0.66*	*18.86*	*1.51*	*92.1*		*1.80*	*0.86*	*20.26*	*1.64*	*89.3*
6. High protein	Standard butter	1	5	1.50	0.51	7.45	1.46	97.3	7	1.73	0.76	9.22	1.66	96.0
		2	5	1.55	0.60	4.46	1.52	98.1	7	2.20	0.78	7.38	2.14	97.4
		3	5	1.65	0.62	8.21	1.60	97.1	7	1.64	0.80	12.59	1.54	94.0
		4	5	1.74	0.60	4.23	1.71	98.2	7	1.98	0.87	8.44	1.91	96.5
Average				*1.61*	*0.58*	*6.09*	*1.57*	97.7		*1.89*	*0.80*	*9.41*	*1.81*	*95.9*
7. Low protein	Special butter spread	1	6	2.43	0.92	10.49	2.28	93.9	4	2.59	1.09	16.24	2.41	93.0
		2	6	1.73	0.67	16.05	1.62	93.8	4	2.07	0.90	19.04	1.90	91.7
		3	6	1.85	0.71	23.25	1.69	91.5	4	2.41	1.02	12.66	2.28	94.6
		4	6	1.98	0.66	28.10	1.79	90.5	4	2.45	1.06	22.50	2.21	90.2
Average				*2.00*	*0.74*	*19.47*	*1.84*	*92.4*		*2.38*	*1.02*	*17.61*	*2.20*	*92.4*
8. High protein	Special butter spread	1	5	2.17	0.85	15.88	2.04	94.1	7	2.41	0.74	18.63	2.27	94.1
		2	5	1.65	0.62	5.04	1.62	98.3	7	1.98	0.89	8.71	1.90	96.0
		3	5	1.64	0.70	13.20	1.55	94.6	7	2.41	1.00	6.34	2.35	97.5
		4	5	1.99	0.71	8.56	1.93	97.1	7	2.52	1.04	7.95	2.44	97.0
Average				*1.86*	*0.72*	*10.67*	*1.78*	*96.0*		*2.33*	*0.92*	*10.41*	*2.24*	*96.2*

[1] Calculated as per rat per day. By "fat absorbed" is meant the difference between fat intake and fecal output.

[2] Not corrected for endogenous fat excretion.

was partially responsible. In addition, when fat intake is taken into account, digestibility (as calculated from the difference between intake and excretion) of all of the fats was decreased in the lower protein groups. This decrease was least serious with the best digested fat (steam lard) and was of greater magnitude with the other fats which exhibited poorer digestibilities. Another observation of importance is the effect of incorporating hydrogenated fats into lard and butter. Calculations of the fatty acid composition of the steam rendered and special stabilized lards gave, respectively: saturated acids, 34.5 and 40.4%; oleic acid, 54.6 and 48.2%; and linoleic acid, 11.0 and 11.4%. This indicates that the special lard contained approximately 6% more saturated fatty acids than the steam rendered lard. Five per cent hydrogenated vegetable fat (iodine value 10) was added to the special butter spread. There was no apparent alteration in the digestibility of the "hardened" butterfat while a marked decrease was observed with the "hardened" lard, especially in the high protein groups.

DISCUSSION

Munk and Rosenheim (von Noorden, '07) have conducted studies in which dogs were fed very low protein diets for 6 or 8 weeks and it was found that digestive powers were seriously upset, finally resulting in death. Fat absorption was most seriously impaired, but the presence of numerous, severe lesions of the intestinal tract led to the conclusion that impaired intestinal functions were the cause of the faulty absorption. Magnus-Levy (von Noorden, '07) quotes studies by Jägerroos and by Chittenden in which no sign of decreased fat digestibility was evident in experimental animals and humans which were maintained on low protein diets. Coffey, Mann and Bollman ('40) measured fat excretion by dogs fed different types of basal diets and found only a slight variation (range 2.11 to 3.99% of the ingested fat excreted in the feces) in the percentage of fecal loss of fat; their high protein diet gave the lowest digestibility, but in this study the fat content of the diets was not constant.

In the experiments reported here the rats were maintained from the time of weaning on diets containing the specified amounts of protein. They were all in good condition and did not exhibit any gross evidence of intestinal pathology. The effects noted were probably not due to lesions of the intestinal tract, but post mortem examinations have not been performed. It is possible that the decreased fat absorption was induced through long continued feeding of the experimental diets and would not be observed in short time studies. The explanation for the

decreased absorption remains obscure. The possibility of a carbohydrate as well as a protein influence upon fat absorption must be given some consideration for decreases in the protein content of the diets were compensated for by corresponding increases in sucrose. An inhibition of fat absorption brought about by increasing the carbohydrate content of the diet does not appear logical, but Briggs, Heller and Wall ('40) have noted that the inclusion of molasses in an oat or corn-alfalfa ration for lambs lowered fat digestibility by 17–18%.

Magnus Levy (von Noorden, '07, p. 55) has presented evidence of an enhanced digestibility with increases in the amount of fat in the diet. Hoagland and Snider ('43), working with rats, have found this to be true for certain hard fats of low digestibility but not for highly digestible oils. In the present communication evidence is given that another dietary ingredient influences fat digestibility. Both the fat and protein contents of the basal diet may remain well within normal nutritional limits and yet exert definite effects upon fat digestibility. Certainly if digestibility measurements from different laboratories are to be compared, a standardized diet must be employed.

The comparisons of digestibilities of lard and butter to which hydrogenated fat has been added are of interest in connection with the use of specially developed "hard fats" which will remain solid at elevated temperatures. The increased fecal loss shown by the blended lard is to be expected if hydrogenation produced much stearic acid. More difficult to understand is the lack of effect when hydrogenated fat was added to butter. The fact that normal butterfat has a relatively low digestibility may have some bearing. One other possibility is that the unique fatty acids in butterfat in some manner counteract the poor digestive characteristics of the hydrogenated fat.

SUMMARY

Comparisons of fat digestibilities in rats receiving diets containing approximately 14 or 30% protein have shown that the lower protein intake is associated with a lower fat digestibility. A well absorbed fat such as lard is influenced only slightly while more poorly digestible fats such as lard containing hydrogenated fat and butterfat are more seriously affected.

The differences in digestibility that are brought about by varying the protein intake emphasize the importance of standardizing the basal diet for all measurements of fat digestibility.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Profs. W. B. Combs and S. T. Coulter of the Division of Dairy Husbandry, University of Minnesota, who furnished the special butter spread that was used in this study.

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A STUDY OF THE DIET OF TWENTY WOMEN IN A MODERATE-INCOME GROUP [1]

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(Received for publication August 25, 1943)

A previous study by the above authors ('43) of the diet of women in a low-income population group has been reported. This study was made by assaying food samples which duplicated in kind and amount the food actually consumed during a day. In the present study this same method was used for a moderate-income group in order to obtain supplemental data on the nutritive value of food as actually consumed. It was necessary to terminate the study in February when food rationing was instituted so that it is not as extensive as was planned. Before this time diet samples had been collected from twenty women, twelve of whom were young married women with children. These twelve women made up a group comparable to the low-income mothers on whom we previously reported. Of the remaining eight women, four were members of the faculty of the Department of Home Economics and four were advanced students in this department.

EXPERIMENTAL

Selection of subjects and collection of samples

The group of twelve young married women who collected diet samples were, with two exceptions, wives of University professors. They were selected because of their interest in the study and willingness to cooperate. The ages of this group ranged from the late twenties to the early forties, the majority falling between 32 and 37 years. All of these women were considered moderately active. The average number of children per mother was 2. One woman was pregnant; none were lactating. Definite information on income was not obtained, but incomes probably ranged from $2500 to $5000 per year. Instructions for the collection of the samples were the same as were given the low-income group, and the aim as before was the duplication in

[1] This research was aided by a grant from the Williams-Waterman Fund.

quantity and kind of the food consumed daily by the individual. The faculty members and students in the Department of Home Economics were asked to collect diet samples because their understanding of and interest in the project guaranteed the utmost care in collection. It was thought desirable to have a group in which accuracy in this respect was beyond question.

Methods of assay

These were the same as were used in the previous investigation and are described in the report of this study. Assay for thiamine was carried out in the fermentometer following the directions of Frey, Atkin, and Schultz ('42). Microbiological methods were used for the determination of riboflavin, pantothenic acid, and niacin. These are fully described in the University of Texas monograph entitled, "Studies on the Vitamin Content of Tissues, I" ('41). Calcium was determined according to the A. O. A. C. method. The colorimetric method of Koenig and Johnson ('42) was used for phosphorus. In order to check the figure of 5 calories per gram of dry sample which was obtained in the low-income study by bomb calorimeter determinations, another series of these determinations were run. Twenty samples were so treated, and the results showed a variation from 4.875 to 5.4 calories per gram and an average value of 5.1. The value of 5 calories used in the low-income study was confirmed as sufficiently accurate for caloric estimations.

RESULTS

Table 1 presents the data obtained from assay of the diets of the group of young married women (group I). These data are comparable with the results obtained on the low-income group since the groups are similar in age and size of family and differ in income, education, and intelligence. The four women faculty members made up group II, and the results obtained on their diets are given in table 2, as are also the results of the analysis of the diets of the four students that make up group III.

DISCUSSION OF RESULTS

Calorie intakes

In the previous study, it was found that the calorie intakes of women in the low-income group were startling low, ranging from 620 to 2050 calories per day and averaging 1145. These women were classed as sedentary on the basis of their observed activity. The average intake was about half the 2100 calorie allowance for sedentary women,

TABLE 1

Nutritive value of diets of women on moderate income level (group I) and comparison with similar values for low-income level.

SUBJECTS	ENERGY	PROTEIN	CALCIUM	PHOSPHORUS	RIBOFLAVIN	PANTOTHENIC ACID	NIACIN	THIAMINE
	Calories per day	*g./day*	*g./day*	*g./day*	*mg./day*	*mg./day*	*mg./day*	*mg./day*
M_1	1865	74.2	1.09	1.54	3.05	7.25	10.34	.65
C_1	1215	...	...	.71	1.18	3.80	5.51	.41
L	1700	...	...	1.07	1.45	4.21	7.35	.75
C_2	1985	53.4	.98	.99	2.09	6.47	9.87	1.04
M_2	2445	79.9	1.63	1.74	2.73	8.48	9.07	1.16
B_1	2120	73.5	1.29	1.09	1.72	3.90	9.65	1.14
P	1885	74.6	1.26	.80	1.89	3.97	10.00	1.11
H_1	1570	59.7	.65	.61	1.06	2.81	9.62	.82
H_2	1405	54.6	.96	.84	1.65	6.22	11.44	.76
B_2	1095	38.4	.40	.59	.96	2.93	6.68	.25
F	1195	40.3	.53	.59	1.24	4.14	9.62	.55
K	1530	49.4	.47	.58	1.55	3.41	9.70	.44
Average	1667	59.8	.93	.93	1.71	4.79	9.07	.72
Low-income Group	1145	33.8	.38	.72	.78	2.41	4.13	.49
Percentage Increase	45%	77%	145%	30%	120%	99%	120%	47%

TABLE 2

Nutritive value of diets of four faculty women (group II) and four University students (group III)

SUBJECT	ENERGY	PROTEIN	CALCIUM	PHOSPHORUS	RIBOFLAVIN	PANTOTHENIC ACID	NIACIN	THIAMINE
	Calories per day	*g./day*	*g./day*	*g./day*	*mg./day*	*mg./day*	*mg./day*	*mg./day*
Group II								
L	1900	61.9	1.17	1.19	1.68	6.65	9.02	1.00
W	1605	53.3	.73	.89	1.88	5.92	10.71	1.00
S	1595	58.8	.76	.86	2.56	6.15	8.31	1.08
T	1680	57.1	.98	.84	1.61	4.49	6.78	.71
Average	1720	57.8	.91	.94	1.93	5.80	8.71	.95
Group III								
F	2020	79.4	...	1.14	2.49	5.37	9.60	.86
M	1900	69.0	.93	.91	1.91	4.61	10.79	.98
H^1	1970	67.4	.90	1.20	2.97	7.40	8.79	1.10
H^2	1795	60.1	1.07	1.01	2.53	5.45	8.80	1.12
Average	1920	69.0	.96	1.07	2.47	5.71	9.49	1.01

recommended by the Committee on Foods and Nutrition, National Research Council. The present moderate-income group of women (group I, comparable to the low-income group of last year) had an average calorie intake of 1667 with a range of 1095 to 2445. While the average intake is 45% above that of the low-income groups, it is still far below the recommended allowance. Most of the women in group I should, in the opinion of the investigators, be classed as moderately active, and, if so classified, the average intake is 66% of the recommended allowance of 2500 calories. It should also be pointed out that, in group I, only two of the women had intakes above the allowance recommended for a sedentary woman and only one approached that for a moderately active woman.

The intake of the women in group II averaged 1720 calories per day, and was slightly higher than that of group I, but the group of students (group III) had a considerably higher average intake, 1920 calories. In no case, however, does the average intake closely approach the recommended allowance for a sedentary woman, i. e., 2100 calories.

There was a lack of correlation between body weight and calorie intake in group I. The two women with the two lowest intakes were inclined to overweight while the one with the highest intake was extremely thin. The other members of group I and all of the women in groups II and III were in the normal range of weight for height.

Basal metabolism determinations were made for thirteen of the twenty women comprising the three groups. The results are given in table 3, together with the calorie intake and the percentage deviation of total calories from the basal. Two of the women had intakes slightly below basal requirements. The percentage increase over the basal varied from 6 to 81%. While all of the basals were within a normal range, all were on the minus side, determinations showing a range of –5 to –15%. A long series of basal determinations made through a period of years on presumably normal students in the University showed an average variation of —8%. The basal metabolism of the women tested was considered not unduly low for this part of the country, although it is possible that there is some correlation between the low basals and the low calorie intakes.

In a dietary study made by Youmans ('42) on a low-income group, as many as a third of the group had calorie intakes that were less than 30% above basal requirements. The same proportion holds true for the present group.

As a result of the study of the low-income group the possibility that the recommended allowances for calories had been placed too high was suggested, and the lack of correlation between calorie intake and body weight was pointed out. The results obtained on the moderate-income group of women emphasize this possibility and indicate again a lack of correlation between body weight and calorie intake.

TABLE 3

Basal calorie requirements, actual calorie intakes, and percentage deviations.

SUBJECT	BASAL CALORIE REQUIREMENTS	CALORIE INTAKE	PERCENTAGE DEVIATION
Group I			
C_1	1099	1215	+ 11
L	1362	1700	+ 26
M_1	1351	2445	+ 81
P	1230	1885	+ 53
H_2	1319	1405	+ 6
B_1	1119	1095	− 2
F	1356	1195	− 13
Group II			
L	1285	1900	+ 48
W	1111	1605	+ 43
S	1216	1595	+ 31
T	1285	1680	+ 31
Group III			
F	1168	2020	+ 73
M	1298	1900	+ 54

Protein intakes

The average protein intakes for groups I and II were practically the same and were very close to the allowance recommended for women, i. e., 60 gm. The protein intake of the student group was somewhat higher, as would be expected from the higher calorie intake. The average protein intake of group I was 77% higher than for the comparable low-income group. Samples were not available for two of the twenty women studied, but, of the eighteen determinations made, only three were more than 10% below the 60-gm recommended allowance.

Mineral intakes

It is interesting that in all three of the groups of women studied this year the calcium and phosphorus intakes were nearly the same

and were approximately 1 gm. each. This contrasts markedly with the results obtained on the low-income group for which an average intake of 0.38 gm. of calcium and 0.72 gm. of phosphorus was indicated. Undoubtedly much larger amounts of milk and green, leafy vegetables were consumed by the higher income group, and it is probably the difference in the consumption of these two foods that accounts for the difference in mineral consumption of these two groups. Phosphorus is not included in the new recommended allowances, but the old "standard allowance" of 1.32 gm. per day per person is approximately one-third more than was furnished by the diets of the present group and two-thirds more than was indicated for the low-income diets. It is difficult to account for the low phosphorus content of these diets, especially in view of the high calcium intake for the moderate-income group. Usually the phosphorus content of a diet is higher than the calcium content, but this was not true for the present group.

Vitamin intakes

As in the previous study assays were made for four of the vitamins: thiamine, riboflavin, niacin, and pantothenic acid. The results of the vitamin assays for group I, together with the corresponding figures for the low-income group are given in table 1. Pantothenic acid intake was twice that indicated for the low-income group, while riboflavin and niacin intakes were more than doubled; thiamine intake, however, was only 47% greater. The better choice of food by the higher-income group is shown by the fact that, while the calorie intake was increased only 45% over that of the low-income group, the intake of all other nutrients, with the exception of phosphorus and thiamine is very much greater than 45%. Figures for the student and faculty groups (tables 2 and 3) show 20–30% larger intakes than for group I of all vitamins except niacin. The intake of niacin varied only slightly for the three groups.

Using the recommended allowances for a moderately active woman for comparative purposes, it may be shown that the average riboflavin intake for group I was 80% of the recommended allowance of 2.2 mg., that for group II, 90% and that for group III, 112%. Similar figures for niacin (recommended allowance, 15 mg.) are 60%, 58% and 63%; for pantothenic acid (using the estimate of 10 mg. suggested by R. J. Williams ('41) 48%, 58%, and 57%; for thiamine (recommended allowance, 1.5 mg.) 48%, 63%, and 70%. Only in the case of riboflavin is a satisfactory intake indicated. This was achieved by a large

milk consumption. As another illustration of the more nearly satisfactory intake of riboflavin it may be pointed out that while 50% of the twenty women studied have riboflavin intakes above 1.8 mg. (recommended allowance for a sedentary woman) none of the women have intakes of thiamine or niacin equal to the allowance for a sedentary woman. It was found, for the low-income group, that the intakes of niacin, thiamine and riboflavin were approximately one-third of the allowance for a sedentary woman while the intake of pantothenic acid was about one-fourth of the suggested 10 mg. standard.

SUMMARY

In estimating the deficiencies of the diet of a group of women on a moderate income, the recommended allowances for a moderately active woman have been used. For a similar purpose, but for a low-income group, the allowances for a sedentary woman were employed. This is in accordance with the opinion of the investigators concerning the activity of the subjects.

The general conclusions in regard to the low-income group were that calorie intakes were from one-half to three-fourths of the recommended allowance; that the average intakes of thiamine, niacin, and riboflavin were approximately one-third and the intakes of protein, calcium, and phosphorus approximately one-half of the allowance recommended. Pantothenic acid was only about one-fourth of the 10 mg. suggested by R. J. Williams ('41) as representing adequacy for this vitamin. Similar conclusions for a comparable group of women on a moderate income level, were that calorie intakes varied, on the whole, from one-half to four-fifths of the allowance recommended, and that the average intakes of protein and calcium indicated no deficiency in these nutrients. A slightly deficient intake of phosphorus is apparent. The average intake of riboflavin was four-fifths, that of niacin three-fifths, and that of thiamine one-half of the allowances recommended for these vitamins. Pantothenic acid intake was a little less than half the amount suggested as representing adequacy. If the allowances for a sedentary woman are used, for the moderate-income group, the data indicate 80% adequacy in calories, 95% in riboflavin, 75% in niacin, and 68% in thiamine. The increase in calorie intake in the moderate-income group was small in comparison with the increase of the other nutrients with the exception of phosphorus. The reasons for believing that the calorie intakes obtained in the present study emphasize the possibility of a too generous recommended allowance have already been discussed.

It was not possible to determine the nutritional status of the subjects of this study as no one competent to make the necessary physical examinations was available.

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STUDIES ON VITAMIN E DEFICIENCY IN CHICKS[1]

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FIVE FIGURES

(Received for publication August 23, 1943)

The two main symptoms of vitamin E deficiency in chicks are exudative diathesis and encephalomalacia. They may appear separately or concomitantly depending upon the composition of the diet. The experiments reported here were designed to examine the manner in which modification of various components of vitamin E deficient diets may influence these symptoms, in the hope that such observations might throw additional light upon the physiological rôle of vitamin E.

In order to evaluate the results of these studies it seems necessary to outline the chief characteristics of these two symptoms and the existing state of our knowledge concerning them.

Exudative diathesis, a condition in which plasma exudes from the capillaries, appears as massive accumulations of fluid in the subcutaneous tissue or as edema of muscles and connective tissue more generally (Dam and Glavind, '38).[2] The affected tissue, especially the adipose tissue, is usually reddish due to fine diffuse hemorrhage. Microscopically there is edema, slight extravasation of red cells and migration of white cells in the affected tissue, and a mild eosinophilia (Dam and Glavind, '39b). The extravasated fluid, which is often greenish from decomposition products of hemoglobin, has approximately the same composition as that of normal plasma. An augmented capacity of the tissue to take up trypan blue intravenously injected (Dam and Glavind, '40) suggests an increased permeability of the capillaries. Since the specific

[1] Aided by a grant from the Josiah Macy, Jr. Foundation.

[2] The fact that vitamin E is the factor which protects against the exudative diathesis has been well established (Dam and Glavind, '39a, '39b; Bird and Culton, '40; Dam, Glavind, Prange and Ottesen, '41). A level of 10 mg. of d, l-α-tocopherol acetate mixed with 100 gm. of the diet always affords sufficient protection. β-tocopherol is much less active against this disease than α-tocopherol. During the study of the distribution of the anti-exudate factor in foods it was found that the lipid of rose hips (Rosa rugosa) is approximately ten times as rich in vitamin E as wheat germ oil (Dam, Glavind, Prange and Ottesen, '41).

gravity of the serum is normal [3] it seems unlikely that a decrease in serum proteins contributes to the appearance of the exudates. Plasma prothrombin and the thromoplastin in the affected tissue are normal. There is no decrease in osmotic resistance of the red cells, no increase in sedimentation rate, and no elevation of body temperature (Dam, unpublished studies).

Although individual chicks of an experimental group as well as different shipments of chicks may vary somewhat with respect to the appearance and severity of the symptoms, the basal diet 182 (table 1) used in most of the present studies usually produces exudates within 14 to 30 days in chicks 1 day old at the beginning of feeding. The exudates generally appear first in adipose tissue, later affect muscles, and are rare in the pericardial and peritoneal cavities. They may disappear spontaneously and recur later in the same chick with no change of the dietary procedure. Absorbed exudates usually leave a buff coloration in the adipose tissue, and sometimes leave a necrotic degeneration of breast and leg muscles leading to permanent invalidism. Severe exudates may be associated with deep and forced respiration. Encephalomalacia occurs occasionally. Chicks grow somewhat subnormally on this diet, attaining weights of 120 to 160 gm. in 4 weeks.

Chicks showing spontaneous disappearance of the symptoms and continued for longer periods on the diet often die with a perforating ulcer of the gizzard, which is unrelated to vitamin E and can be prevented by other modification of the diet.

Using a somewhat different type of diet (table 1), Bird and Culton ('40) report the exudative diathesis to be associated with a considerable accumulation of fluid in the pericardium and peritoneal cavity, indicating that the degree and localization of the exudation may be influenced by the makeup of the diet. The addition of 5% cod liver oil to the Bird and Culton diet has produced, in the author's laboratory, essentially the same symptoms as observed when chicks were fed the exudate-producing diet 182.

Encephalomalacia, the other symptom, was first observed by Pappenheimer and Goettsch ('31).[4] Chicks reared on their diet 108 (table 1)

[3] Fasting values of 1.0154, with a standard deviation of 0.0006, for five 40-day-old chicks receiving the exudate-producing diet, compared with 1.0157, with a standard deviation of 0.00015, for ten 34-day-old chicks on a commercial chicken diet.

[4] The evidence for the relation of encephalomalacia to vitamin E deficiency rests on the finding of a protective factor in the non-saponifiable fraction of various lipids of vegetable origin (Goettsch and Pappenheimer, '36). When synthetic d, l-a-tocopherol became available this substance was found to protect (Dam, Glavind, Bernth and Hagens, '38; Pappenheimer, Goettsch and Jungherr, '39).

become atactic, may fall when trying to walk, get cramps and carry out uncoordinated movements with legs, wings and neck. Frequently they lie on the side with legs stretched or assume a sitting position with the back supported against the wall of the cage. Upon touching, violent movements with legs ("bicycling") and wings occur. In milder cases the birds are only slightly atactic or sit with the body raised and slightly

TABLE 1

	BASAL DIET NO. 32 [1]	BASAL DIET NO. 182 [1]	BIRD AND CULTON'S DIET [7]	PAPPENHEIMER AND GOETSCH DIET 108 [9]	BASAL DIET NO. 190
Casein, unextracted	...	...	...	22.8	...
Casein, alcohol-extracted	15	15	...	...	15
Skimmed milk powder	...	...	54	16.7	...
Dried yeast, unextracted	...	...	...	5.6	...
Dried yeast, ether-extracted	10 [2]	10 [2]	...	...	10 [2]
Corn starch, unextracted	...	...	...	22.2	...
Corn starch, alcohol-extracted	...	...	...	...	24.6
Sucrose	59.8	54.6	44	...	...
Gelatine	8	8	...	...	...
Gum arabic	5	5	...	...	5
Salt mixture	2 [3]	7.2 [3]	1.1332 [5]	7.2 [10]	7.2 [6]
Ground limestone	...	..	1	...	...
l-cystine	0.1	0.1	...	...	0.1
Choline chloride	0.1	0.1	...	...	0.1
Lard	...	...	...	23.3 [4]	30 [4]
Cod liver oil	5 [4]	5 [4]	fed 3 × weekly	2.2 [4]	...
Vitamin K substitute	1 mg.[8]	1 mg.[8]	...	...	1 mg.[8]
Vitamin A, D concentrate	...	...	...	...	1 drop 2 × weekly [11]

[1] This diet is deficient in the peptic ulcer factor (cf. text).

[2] Fleischmann type 2019. Ether-extraction hastens the onset of the symptoms considerably when this type of yeast is used. The Carlsberg yeast used in Copenhagen apparently needed no ether-extraction.

[3] Salt mixture no. 3. 2 gm. consists of: $CaCO_3$ 1457.8 mg., $MgCO_3$ 72.89 mg., NaCl 320.5 mg., Ferric citrate 116.5 mg., Diiodotyrosine 0.018 mg., $CuSO_4$, $5H_2O$ 10.92 mg., $MnSO_4$, $4H_2O$ 21.84 mg.

[4] Added fresh to the diet every day.

[5] 2-methyl-1, 4-naphthohydroquinone diacetate or the tetra-sodium-salt of 2-methyl-1, 4-naphthohydroquinone-diphosphoric acid ("Synkavite, Roche").

[6] Consists of 7.16 gm. of McCollum's salt mixture no. 185, 0.966 mg. KI, 9.93 mg. $CuSO_4$, $5H_2O$ and 39.8 mg. $MnSO_4$, $4H_2O$.

[7] This diet should be supplemented with vitamin K.

[8] Consists of NaCl 1 gm., $MnSO_4$, $4H_2O$ 0.012 gm. and $CuSO_4$, $5H_2O$ 0.0012 gm.

[9] This diet should be supplemented with vitamin K and $MnSO_4$. It gives a marked perosis when fed without added manganese. In the formula given in table 1 the roughage (10% paper pulp) has been omitted, because it has no influence on the experiments.

[10] McCollum's salt mixture no. 185.

[11] Consists of: vitamin A concentrate from fish liver oil (containing 10^6 units per gram) 0.300 gm., vitamin D concentrate (Delsterol, containing 200,000 chick units per gram) 0.200 gm., oleic acid 24,500 gm. The concentrates were obtained from Distillation Products, Inc., Rochester, N. Y. 1 drop (29 mg.) represents 350 units A and 46 chick units D.

swaying. Forced breathing may be apparent. Localized or diffuse areas of edema, fine hemorrhages, and necrosis are usually seen in the cerebellum or the cerebrum. The extent or magnitude of these lesions is not always related to the severity of the symptoms observed. According to Pappenheimer and Goettsch ('31) the cerebellar lesions are characterized histologically by edema, with disorganization of the fibrillar and cellular elements, degeneration of the Purkinje cells and those of the granular layer, small hemorrhages scattered in both white and gray matter, and hyaline thrombosis of capillaries in and around the necrotic areas. The authors stress the capillary thrombosis as the primary cause of the necrotic lesions.

As in the case of exudates, spontaneous recovery with or without recurrent attacks, a buff coloration persisting for some time at the site of earlier lesions, and subnormal body growth are common phenomena in chicks fed the encephalomalacia-producing diet 108; while exudative diathesis rarely appears and no peptic ulcers occur. The water content of the affected parts of the brain is increased (Pappenheimer, Goettsch and Jungherr, '39). A reported decrease in cholesterol content (Adamstone, '41) must be taken with some reservation since the values given for normal chick brain are considerably lower than those generally recorded for brain tissue.

METHODS OF RECORDING SYMPTOMS

One-day-old white leghorn chicks, ten in each experimental and in each control group, were reared in ordinary brooders with raised screen floors and inspected daily, including Sunday, for the appearance of symptoms. The latter are recorded in the graphs as follows. Abscissae indicate age in days, ordinates the number of animals showing symptoms within a given number of days; giving one stair-case curve for exudate (full line) and another for encephalomalacia (broken line), each beginning from the bottom line. In order to give a more accurate representation of the development of the symptoms, exudates are graded by recording a reddishness of the fat tissue which is visible through the skin but not yet accompanied by a palpable exudate as "½ exudate", and adding another "½ exudate" for the same chick when a palpable exudate appears. One animal can be represented by only 1 unit on the ordinate since it is only the beginning of the symptom (eventually divided in two stages) and not its duration or recurrence which is recorded. Animals dying without showing exudate or encephalomalacia are recorded by full lines and broken lines, respectively, moved

down 1 unit from the top (or ½ unit if the animal has had "½ exudate"). The end of each experiment is indicated by a black triangle. In only a few instances were symptoms found at autopsy which could not be observed in vivo. Experiments carried out simultaneously are grouped together in one series. Weight charts are not given but in cases where changes of the diet caused marked changes in body weight specific mention of this effect is made in the text, since one might expect that slow growth would retard the symptoms and rapid growth accelerate them.

FACTORS AFFECTING THE APPEARANCE OF EXUDATES

The salt mixture

The earlier experiments on exudative diathesis (Dam and Glavind, '38, '39a, '39b) were carried out with a lower salt content than that indicated in diet 182; namely, 2% of salt mixture no. 3 (cf. diet 32, table 1), which only approximately supplements the inorganic constituents of 10% yeast and 15% casein. As shown in series 1, the symptoms came much later with such a diet (exp. 3) than with the basal diet 182 containing 7.2% of McCollum's salt mixture no. 185 supplemented with Cu and Mn (exp. 1) or after the addition of sufficient NaCl to diet 32 to give the mineral constituents about the same osmotic concentration as in diet 182 (exp. 4; see also Dam and Glavind, '42).[5] The accelerating effect of NaCl, also observed by Bird ('43), is in agreement with the common clinical experience that salts which tend to enter the extracellular fluids enhance a tendency to edema. On the other hand, the addition of KCl (a salt which does not accumulate in the extracellular fluid) in the same molecular concentration had a slight retarding effect (exps. 8 and 9, series 2), which may or may not be of significance.

In experiments not recorded in the graphs, addition of 5% NaCl to the low salt diet 32 at the fifty-second day of feeding regularly precipitated (within 3 days) pericardial exudates not observed in those members of the group given no additional NaCl. A few instances of such exudates were induced by 5% NaCl even when the diet contained amounts of d, l-α tocopherol (10 mg. %) usually sufficient to prevent exudative diathesis. The pericardial type of exudate appearing rapidly after the feeding of high NaCl is definitely unlike the exudative symptoms developing more slowly on diets containing a more normal salt content.

[5] It will be noted that the experiments recorded in series I took a much longer time than all the following experiments. The reason for this is that in the first series of experiments the yeast was not extracted with ether.

Effect of histamine, etc.

Addition of traces of histamine (25 mg. per kilogram) to the exudate-producing diet with low salt content accelerated the symptom (exp. 5), yet single intravenous injections of shock-producing but sublethal doses of histamine (1 ml. of a 1.25% solution in 0.9% saline) or peptone (5.5 ml. of a 20% solution in 0.9% saline) per 300 gm. body weight failed, even though a transient edema was produced by peptone injection. Addition of histamine (100 mg. per kilogram of diet) did not accelerate the symptoms when there was no fat in the diet.

Symptoms in birds receiving the low salt diet were not accelerated by pinching wings or legs, or by injecting (subcutaneously, intramuscularly or intravenously) 1–2 ml. of the exudate, or by repeatedly injecting 1 ml. of a solution of 1% casein in alkali adjusted to pH 7.3.

Type of carbohydrate

In other experiments not recorded in the graphs the 54.6% sucrose in diet 182 was replaced by either fructose, glucose, cornstarch, alcohol-extracted cornstarch, lactose or galactose. Sucrose and fructose and perhaps glucose gave somewhat more severe symptoms than cornstarch. The effect of the latter was apparently unaffected by alcohol extraction, which removes about 0.6% of partly fat-soluble material. Neither exudates nor encephalomalacia were observed when the diet contained 54.6% lactose, a sugar which is poorly absorbed from the intestine of chicks (cf. Lenkert and Becker, '39), gives poor growth (average weight 69 gm. at 4 weeks) and causes profuse diarrhea when given in such high amounts. Galactose at the same level induces severe cramps and death within a week, associated with high blood sugar (but without any significant change in fermentable blood sugar or muscle glycogen) and almost complete absence of liver glycogen. However, a moderate amount of galactose (10% for instance) can be incorporated in diet 182 without harm and without influencing the symptoms. During the feeding hours sucrose, fructose, lactose and galactose gave reducing sugars in the urine, collected after ligation of the large intestine just superior to the cloaca and rinsing of the latter.

Type of protein

Alcohol-extracted meat meal has been used instead of alcohol-extracted casein with the same effect (Dam and Glavind, '39b). Although extraction of the casein is not absolutely necessary, it increases somewhat the incidence of exudates. Commercial desiccated

chicken eggwhite, non-extracted or extracted with ether or alcohol, and supplemented with biotin [6] proved unsuitable for the development of exudative diathesis, the reason for which is not clear. Since addition of casein (2%) to the eggwhite diet did not increase the incidence of the symptoms, the role of a foreign protein in their production seems unlikely.

The protein-carbohydrate ratio definitely influences the tendency for the exudates to appear. With cornstarch replacing sucrose in the basal diet 182, a casein-cornstarch ratio of 15% : 54.6% (exp. 10) caused an earlier onset of exudates than a ratio of 7.5% : 62.1% (exp. 11). It is possible that retarded growth on the low protein diet, giving an average weight of 74 gm. at 4 weeks compared with 158 gm. for the diet with 15% casein, contributed to the slower development of the symptom, but a casein-starch ratio of 35% : 34.6% likewise retarded the exudates (exp. 12) even though growth was slightly improved (to 166 gm. at 4 weeks). There is apparently a certain protein-carbohydrate ratio which is optimal for the development of the symptom. As was the case in the experiments with the salt mixture, this finding may also have its parallel in clinical medicine where it is common experience that a low carbohydrate diet tends to counteract a tendency to edema.

How far the yeast or other natural source of the B-complex such as skimmed milk powder can be replaced by a mixture of the known B-vitamins without influence on the symptom remains to be investigated.

Type of fat

The fat content of the diet exerts a marked influence on the appearance of both types of symptoms. Their reported acceleration by fat (Dam, Glavind, Prange and Ottesen, '41), attributed to the highly unsaturated fatty acids present (Dam, '43), is amply confirmed by the present studies (series 5). Replacing cod liver oil in diet 182 with a concentrate of vitamins A and D [7] caused no symptoms (exp. 15), whereas with 5% cod liver oil (exp. 16) and the fatty acid fraction of 5% cod liver oil (exp. 17) the symptoms came as usual; the non-saponifiable fraction having no such effect (exp. 18). Also ineffective was 5%

[6] Smaco no. 200, from S.M.A. Corporation, Chagrin Falls, Ohio.

[7] In the experiments recorded in series 4 all groups of chicks received concentrates of vitamins A and D by pipette twice weekly, the amount corresponding to 68 units of A and 5 chicken units of D dissolved in 7 mg. cod liver oil per animal per day. In all other series which included diets without cod liver oil a similar or a higher amount of A and D was given in the same manner, the solvent being either cod liver oil or oleic acid in the amount mentioned. It appeared to be of no importance which solvent was used as long as the amount was very low.

commercial oleic acid freed from traces of non-saponifiable matter (exp. 20), whereas fatty acids from linseed oil similarly purified resembled cod liver oil fatty acids in their effect (exp. 19). The observation, not recorded in the graphs, that the addition of 5% cod liver oil to the diet of chicks reared for 4 weeks on diet 182 minus this oil precipitated exudates 4 to 7 days later indicates that a certain time is required to induce the capillary damage. The symptoms produced by 5% lard were slower in their appearance but no less severe than those induced by 5% cod liver oil (exps. 13 and 14). It has further been observed that 5% commercial C_{20} unsaturated fatty acids [8] (iodine value 235) also accelerate exudates even though they retard body growth (to 83 gm. at 4 weeks). There seems to be little doubt, therefore, that it is the highly unsaturated fatty acids which accelerate the exudative diathesis.[9]

It is well known that cod liver oil exerts a muscular dystrophy-inducing action in rabbits which is accentuated by rancidity changes in the oil and reduced or abolished by hydrogenation (McCay, Paul and Maynard, '38; Mattill and Golumbic, '42; Mackenzie, Mackenzie and McCollum, '41), leading to the hypothesis that auto-oxidation of the fatty acids in cod liver oil destroys traces of vitamin E in the diet (and perhaps also in the body). That cod liver oil exerts a direct damage on tissues seemed unlikely, since muscular dystrophy occurs in rabbits fed an E-deficient diet exceedingly low (0.05%) in animal fats (Mackenzie, et al., '41). Since fat in the diet is prerequisite to the marked development of E-deficiency symptoms in chicks, and since previous studies (Dam, '43) indicate that the exudate-inducing action of fresh cod liver oil is unchanged by slight rancidity but abolished by thorough rancidity, the effect of fats and of rancidity upon dystrophy in rabbits appears to have no parallel in the exudative diathesis in chicks.

Additional studies reported here, carried out under slightly different conditions, further confirm these findings. Without cod liver oil no symptoms occurred (exp. 23). With fresh cod liver oil (iodine value 173) exudates developed as usual (exp. 24). With cod liver oil made thoroughly rancid in the diet by exposing the latter in thin layers for 1 week at 50°C., reducing the iodine value to 62, no symptoms occurred (exp. 25). Separate daily addition of the yeast to the diet after rancidity was developed gave the same result (exp. 26). Likewise, no symptoms appeared when the diet contained cod liver oil which had been heated in a rapid current of air at 100°C. for 1 week, reducing the iodine value

[8] Obtained from Armour and Company, Chicago.

[9] The basal diet 182 minus cod liver oil, which contains only traces of fat in the extracted yeast and casein (Mackenzie et al., '39), has produced exudates in only 3 out of 100 chicks. Two of these exudates were not accompanied by reddening of adipose or muscle tissue.

to 86 (exp. 27). Although in the latter experiment polymerisation of the oil may have occurred and interfered with its intestinal absorption, this series of experiments clearly demonstrates that thorough rancidity of the cod liver oil developing in the diet abolishes its accelerating effect on the symptoms.[10] This effect of cod liver oil (or highly unsaturated fatty acids) on exudative diathesis must therefore be due to changes independent of destruction of traces of vitamin E in the diet through rancidity of the oil. The possibility that rancidity of the oil leads to the formation of a factor which protects against the symptoms is excluded because the addition of 5% fresh cod liver oil to the rancid diet resulted in appearance of exudates just as when no rancidity was present (exps. 28 and 24). The general trend of these experiments is in agreement with the observations that various hydroquinones which may act as antioxidants do not prevent the exudative diathesis in chicks (Bird, '43) and that the antioxygenic activity of a given compound is not a measure of its vitamin E activity (Golumbic, '41). It is also in accord with the fact that cod liver oil has been used as solvent for free tocopherol in experiments with chicks (Bird and Culton, '40) and with rats (Bacharach et al., '37; K. E. Mason, personal communication) without destroying the protective effect of the tocopherol.[11]

Cholesterol and lipotropic substances may influence the effect of cod liver oil on the exudates (Dam and Glavind, '42). Thus the addition of 1% cholesterol to diet 32, which in itself gives a slow development of the symptoms, will cause the symptoms to appear more rapidly, whereas addition of 1.5% inositol or 2% lipocaic [12] to diet 182 resulted in marked retardation. The experiments on which the preliminary report was based are presented in series 1 (exps. 2 and 6), and series 7 (exps. 29 to 31). It was found later that cholesterol had no accelerating effect when cod liver oil was omitted from the diet. It has also been found that mannitol, sorbitol, and dulcitol do not possess the exudate-retarding property of inositol.

[10] On an earlier occasion Dam and Glavind ('39) thought that rancidity might favor exudates because, in a period where the diet unintentionally had become rancid due to lack of cleanness, chicks which received a supposedly vitamin K free diet showed exudates before hemorrhages were observed. However, this must have been due to the fact that the rancidity was not complete and that simultaneous putrefaction of the casein delayed the decline of prothrombin so that the exudates appeared before the hemorrhages.

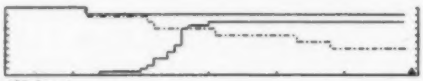
[11] That tocopherol acetate can be incorporated in diets with 5% cod liver oil without losing its protective effect is not surprising because the acetate is less easily oxidized than the free compound.

[12] From Eli Lilly and Co., Indianapolis. The absence of tocopherol from the lipocaic preparation was ascertained by hydrolysis of 2 gm. with 60% KOH in absence of air extraction, washing and evaporating under the same conditions and carrying out the Emmerie and Engel test as described by Dam, Glavind, Prange and Ottesen ('41).

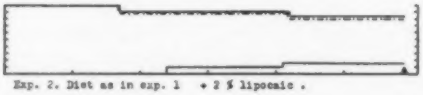
FACTORS AFFECTING THE APPEARANCE OF ENCEPHALOMALACIA

The Pappenheimer and Goettsch diet 108, hitherto used extensively for the study of encephalomalacia, contains large amounts of lard and moderate amounts of carbohydrate in the form of cornstarch. It also contains lactose from skimmed milk powder but, as previously mentioned, this carbohydrate is poorly utilized by chicks and, therefore, can be left out of consideration. With this in view it is possible to modify the exudate-producing diet 182 so that encephalomalacia appears as the chief symptom. Series 9 shows experiments of this kind.

SERIES 1.

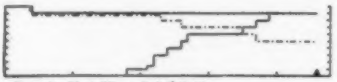


<10 days>
Exp. 1. Diet 182 with unextracted yeast.

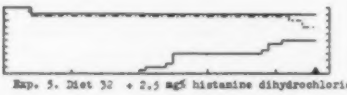


Exp. 2. Diet as in exp. 1 + 2 % lipocaic.

Exp. 3. Diet 32 (2 % salts # 3).



Exp. 4. Diet 32 + 2.9 % NaCl.

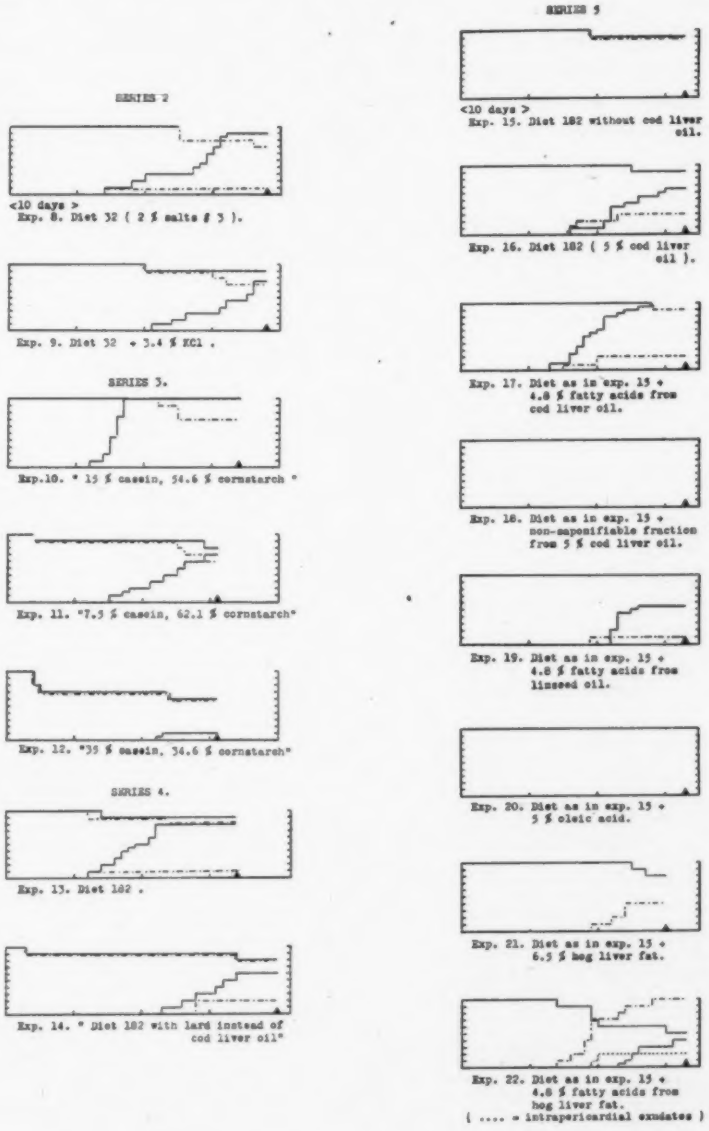


Exp. 5. Diet 32 + 2.5 mg% histamine dihydrochloride

Exp. 6. Diet 32 + 1 % cholesterol.

Exp. 7. Diet 32 with dried hog liver instead of casein.

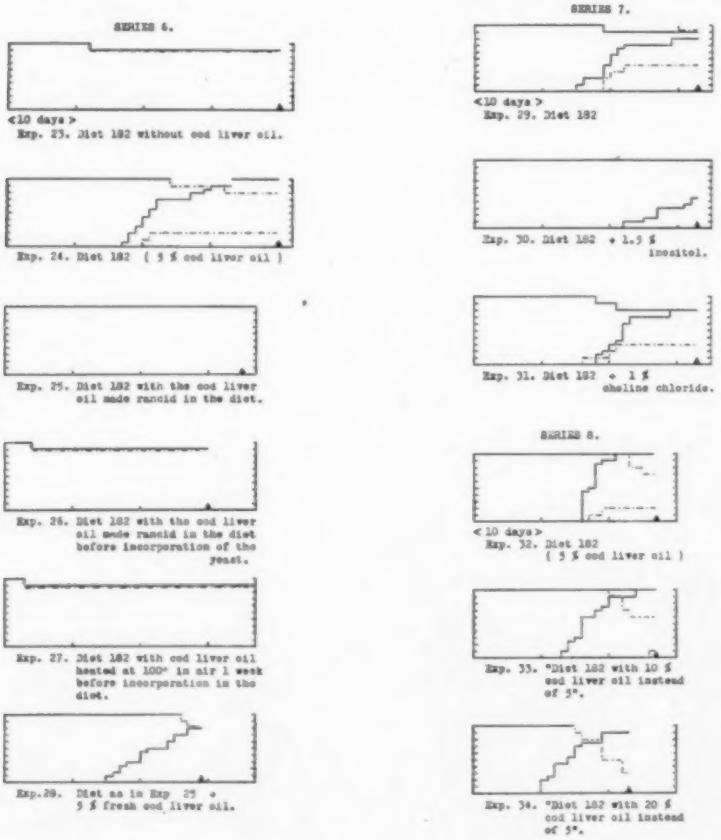
Cod liver oil was omitted in order to make the experiments simpler and the variations of the diet consisted in changing the lard and carbohydrate as indicated in the legend, the other constituents being unaltered.


SERIES 2
<10 days >
Exp. 8. Diet 32 (2 % salts # 3).
Exp. 9. Diet 32 + 3.4 % KCl .
SERIES 3.
Exp.10. * 15 % casein, 54.6 % cornstarch *
Exp. 11. "7.5 % casein, 62.1 % cornstarch"
Exp. 12. "3? % casein, 54.6 % cornstarch"
SERIES 4.
Exp. 13. Diet 182 .
Exp. 14. " Diet 182 with lard instead of cod liver oil"
SERIES 5
<10 days >
Exp. 15. Diet 182 without cod liver oil.
Exp. 16. Diet 182 (5 % cod liver oil).
Exp. 17. Diet as in exp. 15 + 4.8 % fatty acids from cod liver oil.
Exp. 18. Diet as in exp. 15 + non-saponifiable fraction from 5 % cod liver oil.
Exp. 19. Diet as in exp. 15 + 4.8 % fatty acids from linseed oil.
Exp. 20. Diet as in exp. 15 + 5 % oleic acid.
Exp. 21. Diet as in exp. 15 + 6.5 % hog liver fat.
Exp. 22. Diet as in exp. 15 + 4.8 % fatty acids from hog liver fat.
(.... = intrapericardial exudates)

Fat, protein and carbohydrate

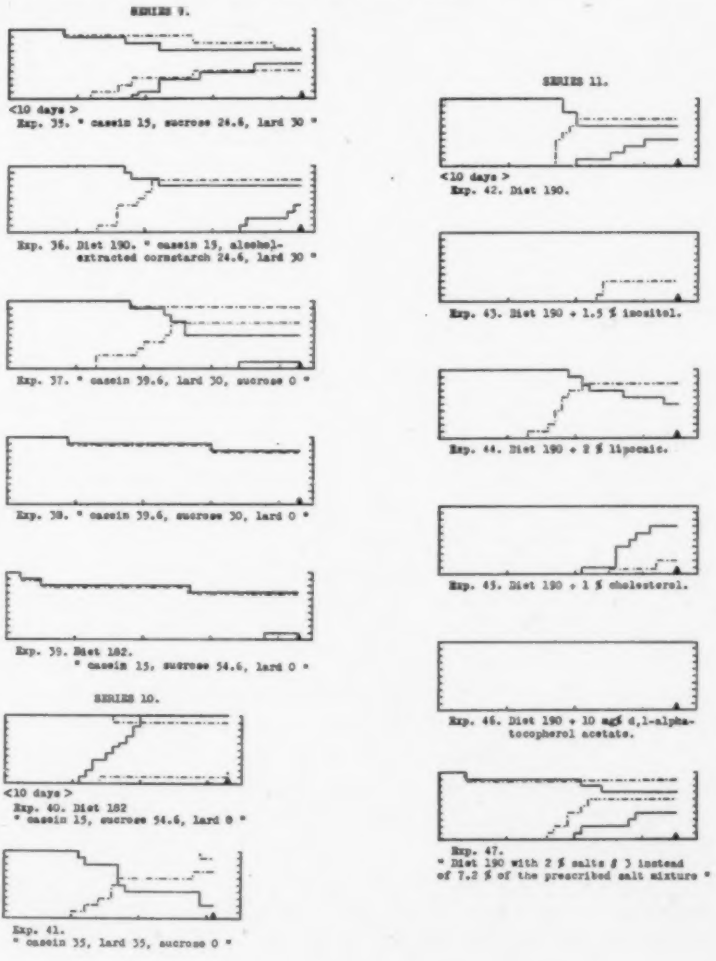
With 30% lard replacing the same amount of sugar, whereby the sugar was reduced to 24.6%, both exudates and encephalomalacia developed about equally; encephalomalacia appeared slightly in advance of the exudates (exp. 35). With 30% lard and 24.6% cornstarch (diet 190, table 1), encephalomalacia was by far the dominating symptom, exudates coming decidedly later (exp. 36). Encephalomalacia was still more favored when all the carbohydrate of the diet containing 30% lard was replaced by casein, the total casein content being 39.6% (exp. 37).

Without lard (exps. 38 and 39) no encephalomalacia appeared but one instance of exudate, rarely seen on "fat-free" diets, did occur (exp. 39). It is interesting to compare the latter with experiments 40 and 41,


SERIES 6.
<10 days>
Exp. 23. Diet 182 without cod liver oil.
Exp. 24. Diet 182 (5 % cod liver oil)
Exp. 25. Diet 182 with the cod liver oil made rancid in the diet.
Exp. 26. Diet 182 with the cod liver oil made rancid in the diet before incorporation of the yeast.
Exp. 27. Diet 182 with cod liver oil heated at 100° in air 1 week before incorporation in the diet.
Exp. 28. Diet as in Exp 25 + 5 % fresh cod liver oil.
SERIES 7.
<10 days>
Exp. 29. Diet 182
Exp. 30. Diet 182 + 1.5 % inositol.
Exp. 31. Diet 182 + 1 % choline chloride.
SERIES 8.
< 10 days>
Exp. 32. Diet 182 (5 % cod liver oil)
Exp. 33. "Diet 182 with 10 % cod liver oil instead of 5".
Exp. 34. "Diet 182 with 20 % cod liver oil instead of 5".

series 10, in which the diets contained 5% cod liver oil. When in diet 182 the sugar was removed and the casein increased to 35%, with the simultaneous introduction of 35% lard, the symptom shifted from exudate to encephalomalacia, the body weight increasing to 265 gm. in 4 weeks.

A similar shift occurred when cod liver oil or its fatty acids in diet 182 (exps. 16 and 17) were replaced by hog liver fat or its fatty acids (exps. 21 and 22), and when dried hog liver replaced casein as the source of protein in diet 32 (exp. 7). Hog liver fatty acids produced intrapericardial exudates in two chicks (exp. 22).


SERIES 9.
<10 days >
Exp. 35. " casein 15, sucrose 24.6, lard 30 "
Exp. 36. Diet 190. " casein 15, alcohol-extracted cornstarch 24.6, lard 30 "
Exp. 37. " casein 39.6, lard 30, sucrose 0 "
Exp. 38. " casein 39.6, sucrose 30, lard 0 "
Exp. 39. Diet 182.
" casein 15, sucrose 54.6, lard 0 "
SERIES 10.
<10 days >
Exp. 40. Diet 182
" casein 15, sucrose 54.6, lard 0 "
Exp. 41.
" casein 35, lard 35, sucrose 0 "
SERIES 11.
<10 days >
Exp. 42. Diet 190.
Exp. 43. Diet 190 + 1.5 % inositol.
Exp. 44. Diet 190 + 2 % lipocaic.
Exp. 45. Diet 190 + 1 % cholesterol.
Exp. 46. Diet 190 + 10 mgs d,l-alpha-tocopherol acetate.
Exp. 47.
" Diet 190 with 2 % salts # 3 instead of 7.2 % of the prescribed salt mixture "

Not only cod liver oil (exp. 16) but also linseed fatty acids (exp. 19) and lard (exp. 14) caused some encephalomalacia when constituting about 5% of the diet, while commercial oleic acid (exp. 20) or thoroughly rancid cod liver oil (exps. 25–27, series 6) did not. Furthermore, increasing the cod liver oil in diet 182 from 5% to 20% (exps. 32–34, series 8) did not increase the incidence of encephalomalacia but may have reduced it.

The data thus far collected show that certain fats will favor exudates whereas others will favor encephalomalacia. Whether this is due to the fatty acids themselves or to trace substances which go into the fatty acid fraction remains to be definitely settled. Encephalomalacia has never been observed, and exudates rarely seen, when fat was not added to the diet. Furthermore, low carbohydrate or the use of a moderate amount of starch as the source of carbohydrate instead of sucrose will depress the tendency for the exudates to appear and favor encephalomalacia, provided sufficient fat such as 30% lard is present.

Cholesterol and lipotropic substances

The experiments included in series 11 demonstrate that both encephalomalacia and exudates were counteracted by 1.5% inositol (exp. 43), only exudates by 2% lipocaic (exp. 44), and only encephalomalacia by 1% cholesterol (exp. 45); whereas 10 mg. % of α-tocopherol prevented both symptoms (exp. 46). Changing the salt mixture to 2% of salts no. 3 (exp. 47) had no significant influence except that growth was retarded (average weight of 126 gm. at 4 weeks, compared to 175 gm. with diet 190 and 200 gm. with diet 190 plus inositol). It is of interest to note that with diet 182 containing 5% cod liver oil, 2% lipocaic likewise suppressed exudates (exp. 2) whereas 1% cholesterol added to diet 32 exerted an accelerating effect upon exudates (exp. 6) which did not occur with diet 190 (cf. exps. 45 and 42). From the foregoing it is apparent that substances influencing fat metabolism will also influence the nature and incidence of the symptoms. Obviously the problems raised by these observations require further investigation with varying amounts of the substances tested.

The failure of the Pappenheimer and Goettsch diet 108 to induce exudates has been explained on the assumption that certain ingredients of the diet provide traces of vitamin E sufficient to prevent this symptom but not adequate to meet a supposedly higher requirement for the prevention of encephalomalacia (Dam and Glavind, '39b). The results of the present studies indicate that the low carbohydrate content of this diet in itself tends to delay or minimize the tendency to exudates.

DISCUSSION

One might ask whether encephalomalacia is not simply due to exudation in brain tissue. Several of the above mentioned facts obviously speak for this view. However, the question is complicated by the fact that there is no complete parallelism between the factors which favor or counteract the two symptoms.

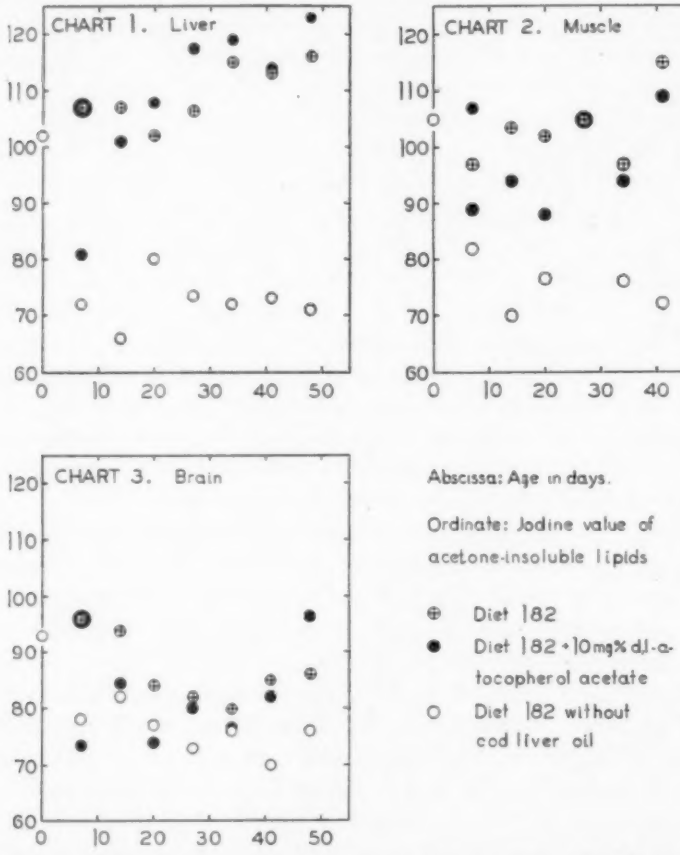
If fats merely destroy vitamin E in the body, it is difficult to explain why some fats favor exudates and others encephalomalacia, and why dietary fat is so essential for the development of symptoms in chicks but not so in rabbits. Certain fatty acids undoubtedly exert a damaging effect on the affected tissues, directly or indirectly but the nature of this mechanism remains to be investigated. Vitamin E deficiency seems not to incur a general inability to metabolize fat as manifested by ketonuria, lipemia or fatty liver. There is some evidence (Dam and Kelman, '42) that a deficiency of vitamin E decreases the ratio of phospholipids to other lipids in the blood plasma of chicks but the difference (20–40%) is not great enough to be of diagnostic value because of the considerable individual variation. The present studies have revealed no significant alteration in tissue lipids of deficient chicks, except for an increase in muscle cholesterol (170 mg. % with diet 182 against 104 mg. % when the diet contained 10 mg. % d, l-α-tocopherol acetate); a finding in harmony with observations on rats (Heinrich and Mattill, '43). Tests for rancidity in the fat tissue were negative.

Sinclair ('32) has shown that in rats the phospholipids take up highly unsaturated fatty acids from the diet. In order to find out whether vitamin E has any gross influence on this process I determined the iodine value [13] of the phospholipids of liver, muscle and brain from chicks reared on diet 182, diet 182 + 10 mg. % d, l-α-tocopherol acetate, and diet 182 without cod liver oil (vitamins A and D concentrate being given to all groups). Charts 1, 2 and 3 show that while there was a considerable difference in iodine value of liver and muscle phospholipids according to whether cod liver oil was given or not, the presence or absence of vitamin E did not cause any significant difference. It has further been shown that the iodine value of the subcutaneous depot fat is not significantly influenced by the presence or absence of vitamin E.

[13] The organs were ground with sodium sulfate immediately after killing by decapitation, the lipids extracted with chloroform at room temperature for 24 hours, evaporated in vacuo at body temperature and extracted six times with acetone. After dissolving the residue in chloroform the dry weight was determined in one aliquot and the iodine value in another, the method being that of Rosenmund and Kuhnhenn as described by Dam ('30).

Regarding the modifying influence of inositol, lipocaic, cholesterol, carbohydrates, and protein-carbohydrate ratios, it may be concluded that this influence is too complicated to be explained by general pro- or anti-oxidant effects.

Some observations relative to the response of rats to diets which produce exudates or encephalomalacia in chicks warrant brief mention here. Rats showed no such symptoms when fed a vitamin E deficient



diet from the time of weaning, even if the amount of cod liver oil in the diet is high. However, a peculiar buff discoloration of the subcutaneous and intraperitoneal fat tissue occurred without any signs of hemorrhage in (a) rats reared for 183 days on diet 182, to which was added 1% cholesterol, 1% NaCl and 60 mg. % of histamine dihydrochloride, and in (b) rats reared for 111 days on diet 182, modified by increasing the

cod liver oil from 5% to 20%. This change of the fat did not occur either in controls fed the same diets + 10 mg. % d, l-α-tocopherol acetate or in rats fed the unsupplemented diet 182 containing only 5% of cod liver oil. Should it be possible to demonstrate that this phenomenon is related to the buff coloration which develops in fat tissue in chicks receiving diet 182, after the reddishness of the fat tissue has disappeared, there would exist a bond of similarity in the response of these two species to vitamin E deprivation. In this connection it may be noted that a hemorrhagic tendency has been observed in the fetus of rats receiving a vitamin E low diet (Mason, '42, '43), which may have some relation to the exudative diathesis in chicks.

Rabbits will develop muscular dystrophy when placed on various vitamin E deficient diets, including the Pappenheimer and Goettsch diet 108, but nothing resembling exudates or encephalomalacia has been observed in them. Pappenheimer and Goettsch ('34) made the surprising observation that diet 108 also produces only muscular dystrophy in ducklings. Ten newly hatched ducklings reared for 33 days in the author's laboratory on the usual exudate-producing diet 182 did not show any definite signs of exudate or encephalomalacia. The reason for this different behavior of different species even when they are so closely related as ducks and chicks has not been explained, but it would not seem unlikely that differences in the fat-metabolizing enzyme system may play a rôle.

Symptoms corresponding to exudative diathesis and encephalomalacia have not been recognized in humans. The possibility that vitamin E might influence the capillary phenomena in certain forms of allergy was examined by Glaser and Dam ('44) with negative results.

SUMMARY

The symptoms of vitamin E deficiency in chicks — exudative diathesis and encephalomalacia — can be accelerated or suppressed by dietary changes which are unrelated to the vitamin E content of the diet.

Purified diets containing no added fat rarely produce exudates and never induce encephalomalacia. Both symptoms are accelerated by highly unsaturated fatty acids.

The fatty acids from cod liver oil, lard and linseed oil and commercial unsaturated C_{20} acids at a level of about 5% in the diet produce exudates as the main symptom, whereas fatty acids from hog liver favor the appearance of encephalomalacia. Oleic acid and thoroughly rancid cod liver oil are ineffective in producing either symptom.

A certain protein-carbohydrate ratio is optimal for the appearance of exudates, and the tendency to exudates is further enhanced by those inorganic salts which tend to accumulate in the extracellular fluid.

High lard (30%) favors encephalomalacia more than exudates when the carbohydrate-protein ratio is low. Inositol (1.5%) counteracts both symptoms. Lipocaic (2%) counteracts only exudative diathesis. Cholesterol (1%) hastens exudates when the diet contains 5% cod liver oil and a low salt content, and counteracts encephalomalacia when the diet contains 30% lard. The iodine value of body phosphatides is influenced by dietary fat independently of the presence of vitamin E.

It is believed that the effect of the different fatty acids consists in a damage of tissue rather than in a general destruction of vitamin E, and that inositol, lipocaic and cholesterol do not act simply through a general pro- or anti-oxidant effect.

The response of different species of animals to diets which produce exudates or encephalomalacia in chicks is discussed.

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